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Assessing the Relationship of Monocytes with Primary and Secondary Dengue Infection among Hospitalized Dengue Patients in Malaysia, 2010: A Cross-Sectional Study

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Assessing the Relationship of Monocytes with Primary and Secondary Dengue Infection among Hospitalized Dengue Patients in Malaysia, 2010: A Cross-Sectional Study

by

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DEDICATION

This thesis is dedicated to my parents, Mark & Christine Klekamp.

Their lifelong unwavering support, encouragement, and love for me, has and continues to be my most treasured memory.

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LIST OF SYMBOLS AND ABREVIATIONS

ADE	Antibody-dependent enhancement
CE	Common era
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
ELISA	Enzyme-linked immunosorbent assay
HLA	Human leukocyte antigen
ICU	Intensive care unit
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
km	Kilometer
NS1	Non-structural protein one
RT-PCR	Reverse transcriptase – polymerase chain reaction
SEAR	Southeast Asian region
WHO	World Health Organization
WPR	Western Pacific region
WWII	World War Two

DEFINITIONS

Defervescence	"Abatement of a fever as indicated by a reduction in body temperature" (WordNet, 2011).
Endemic	"The constant presence of a disease or infectious agent within a given geographic area or population group; may also refer to the usual prevalence of a given disease within such an area or group" (Last, 1988).
Epidemic	"The occurrence in a community or region of cases of an illness with a frequency clearly in excess of normal expectancy" (Chin, 2000).
Hyperendemic	"A disease that is constantly present at a high incidence and/or prevalence and affects most or all age groups equally" (Last, 1988).
Outbreak	"An epidemic confined to a localized area, such as a town or within an institutional setting like a day-care center" (Oleckno, 2002).
Pandemic	"An epidemic occurring worldwide or over a very wide area, crossing international boundaries, and usually affecting a large number of people" (Last, 1988).
Pantropics	"Area between the tropics of Cancer and Capricorn, defined by the parallels of latitude approximately 23°30′ north and south of the Equator" (Farlex, 2011).

ABSTRACT

Dengue, a group of four similar viruses transmitted through the bite of a mosquito, is estimated to infect upwards of 100 million annually in over 100 nations throughout the global equatorial belt. Distribution of global dengue is highly skewed as Southeast Asian and Western Pacific regions endure 75% of the global dengue burden. Similar to other regional countries, Malaysia has been rapidly urbanizing, which has supported a hyperendemic dengue state.

The biological pathway by which dengue infection causes a wide range of clinical manifestations, spanning asymptomatic to life-threatening severe complications, is not comprehensively understood. Historically, severe dengue complications have primarily occurred in children. Consequentially, the majority of the dengue biological pathway research has been conducted on children; however, extrapolation of research findings to adults may be inappropriate as dengue manifestations have differed between age groups. As developing countries undergo epidemiologic transitions and dengue continues to spread geographically to non-endemic regions, youth and adult populations have been subjected to more of the severe dengue burden.

Epidemiology and laboratory-based evidence has supported both memory T-cell and antibody independent enhancement hypotheses to explain the

biological pathway of severe dengue. Both hypotheses employ the central idea that a primary infection alters immune components so that during a secondary heterotypic dengue infection, an individual is more at risk for severe complications.

Monocytes, immune cells that are pivotal in both hypotheses, have been highly examined through *in vivo* and *in vitro* experimentation; however, epidemiological evidence for monocyte involvement is incomplete. The primary objective of the study was to examine if a difference in absolute monocyte count, considering independent risk factors, is present in individuals with primary and secondary dengue infections.

A secondary dengue infection was found to raise absolute monocyte count during the defervescence phase of dengue illness in individuals aged 15 years and older 0.71 ± 0.15 (x10^9) compared to those experiencing primary dengue infection. Gender and distance of study participants' residences from Hospital Ampang were found to be risk factors for the relationship of interest; whereas, age and race were not found to be significant risk factors.

The study helps expand current knowledge of the severe dengue biological pathway with respect to immunological differences between primary and secondary dengue infections. Further research is needed to confirm and expand the findings of this initial study, specifically to include infecting dengue

serotype, education, and socioeconomic status which are known dengue risk factors.

INTRODUCTION

Dengue, a flaviviris transmitted by the bite of a mosquito (*Aedes* genus), is currently estimated to infect upwards of 100 million individuals worldwide annually. There are four serotypes of the dengue virus, each capable of causing a range of manifestations from asymptomatic infection to life threatening complications (i.e. hemorrhage, shock, severe organ impairment). Dengue is an emerging public health threat in over 100 tropical and sub-tropical countries, however, disproportionately burdens Southeast Asia and the Western Pacific nations with 75% of the annual global dengue incidence (WHO/TDR, 2009; CDC, 2011).

The first recorded outbreak of dengue in Malaysia, located in Southeast Asia, occurred in 1901 (Skae, 1902). In the last century, dengue has become hyperendemic across much of Malaysia, meaning the disease is constantly present at a high incidence and affects most of the population (Last, 1988). The financial cost of dengue in Malaysia has been estimated in 2006 at US$12.8 million annually, with 54% allocated for dengue treatment and 46% towards vector control (Suaya, Shepard, & Beatty, 2006). Even with increased vector control efforts by the Malaysian Ministry of Health, the burden of dengue

continues to grow as evident by the highest number of annual dengue mortality recorded to date of 134 individuals in 2010 (MMOH, 2011).

The uncontrolled worldwide epidemics of dengue is cause for alarm; however, until the last half century dengue was a widely unknown, uncharacterized disease with little burden on the human population.

Global Dengue History

Due to disease similarities, the origins of the dengue virus are disputed and are divided into two lines of thinking: African and Asian. The proponents for the African origin point to the current commensal relationship between the dengue virus and the primary vector *Aedes aegypti*, which originates from Africa. The Asian theory of dengue origin points to recent ecological, serological and phylogenetic evidence of *Aedes aegypti* and dengue virus emergence and evolution within Asia to make their ancestral claim (Smith, 1956; Rudnick & Lim, 1986; Wang *et al.,* 2000).

Historical documents clinically describing a dengue-like illness first appear in China from the Chin Dynasty (265-420 Common era (CE)) (Gubler, 1998). A gap of nearly seven centuries passes before descriptions reappear in the French West Indies in 1635 CE (Gubler, 1997). By 1800 CE, many accounts of dengue-like outbreaks along trading routes in the tropical and sub-tropical global belt had been documented. Areas with documented dengue-like outbreaks during this

time period include Cuba, Brazil, Hawaii, East Africa, Syria, Egypt, United States, Indonesia, Panama, Spain, India, Burma, and Philippines (Vasilakis & Weaver, 2008). From the early 18th to 19th centuries, dengue-like epidemics traversed the globe including five known pandemics (Gubler, 1997). While it is impossible to know which of the four dengue serotypes was responsible, it has been hypothesized that only one serotype was responsible due to the lack in severe manifestations that are currently observed to be associated with heterotypic secondary dengue infections (Gubler, 1997).

The wave of scientific breakthroughs in infectious disease research during the early 20th century brought empirical evidence of both the etiological agent and vector for dengue. In 1907, U.S. Army Medical Corps Captain Ashburn and Lieutenant Craig were stationed in the Philippines to determine if dengue was a blood-borne disease transmitted through mosquitoes, as literature of the time suggested (Gubler, 2004). Results of their studies were inconclusive towards the causal vector; however, they were able to determine that the dengue agent was in-filterable (i.e., a virus) (Gubler, 2004). *Aedes egypti* was demonstrated to be the causal vector using human volunteers in 1926; followed by *Aedes albopictus* in 1931 (Vasilakis & Weaver, 2008).

World War II (WWII) is a defining moment in time for dengue. Before WWII, dengue was a little-studied disease which caused a flu-like non-fatal illness that would typically pass within a week. The war brought many changes which altered the environment and demographics, especially in the Asian theater

of war. As the Empire of Japan expanded its regional borders into southern neighboring nations, and upon the United States of America entering the war against Japan, tens of thousands of dengue susceptible individuals flooded into dengue endemic regions. Mass movement of supplies and troops inadvertently transferred dengue across much of the global tropical and sub-tropical belt (Gubler, 1997). As a result of poorly designed urban military and civilian developments, increased in water storage tanks, and discarded war material serving as breeding sites for *Aedes* larvae dengue epidemics in Africa, Mediterranean region, and the Americas, along with a pandemic in the Pacific occurred throughout WWII (Gubler, 1997).

As the effect of dengue on Allied and Axis troops increased, both sides invested resources into dengue research. Dengue virus serotype 1 (DENV-1) was first isolated in Japan in 1943, which was quickly followed by the American isolation of DEN-1 and dengue virus serotype 2 (DENV-2) in 1944 (Sabin, 1952). Further investigations resulted in the development of the haemagglutination inhibition (HI) test (Sabin, 1952). Haemagglutination inhibition allowed for a laboratory diagnosis of dengue infection using “homotypic” immunological responses in primary and secondary dengue infected individuals (Sabin, 1952). These pioneering scientific discoveries permitted dengue to be distinguished from other diseases presenting with similar clinical manifestations.

As the war concluded and troops withdrew, dengue had taken hold in the Pacific and perceptions on the potential threat of dengue soon changed. During

the post-war period, displaced war refugees concentrated in already crowded urban cities. These rapidly expanding urban centers lacked infrastructure for sanitation and water distribution capabilities, which lead to a rise in household water storage tanks (Gubler, 1997). Under these ideal breeding conditions *Aedes aegypti* populations soared and continued to spread geographically through military and civilian transport vehicles (Gubler, 1997). The Pacific was now experiencing multiple co-circulating dengue serotypes contributing to the first recorded dengue hemorrhagic fever (DHF) epidemic in Manila, Philippines in 1953 (Rigau-Perez *et al.,* 1998). Both dengue virus serotypes 3 and 4 (DENV-3 and DENV-4) were isolated for the first time during the DHF outbreaks in the 1950s (Rigau-Perez *et al.*, 1998). Prior to the 1953 dengue outbreak, DHF-like symptoms had been described in two historical accounts: United States in 1780 and Greece in 1927 (Vasilakis & Weaver, 2008). Unlike the 1953 DHF outbreaks, the historic outbreaks are thought not to have been a significant threat to public health. Dengue fever epidemics have remained largely unobserved in the last 25 years due to the disease endemic nature in Asia; however, DHF epidemics have spread throughout much of the Pacific region mainly effecting children (Pinheiro & Corber, 1997). Dengue surveillance in Malaysia corroborates the epidemiological patterns of dengue in Asia. From 1987-1991, dengue fever incidence continually increased from 10.4 to 32.7 per 100,000 individuals (Poovaneswari, 1993), and prior to 1982 over 50% of dengue mortalities were in children (George & Lam, 1997).

Historic dengue epidemiology in the Americas is in stark contrast to that experienced in Asia. The first record of a dengue-like outbreak occurred in Martinique and Guadeloupe in 1635, followed by epidemics and pandemics mirroring similar levels seen in pre-WWII Asia (WHO, 1996). However, in 1947, in an attempt to rid Yellow Fever Virus from the Americas, an *Aedes aegypti* eradication plan was implemented (WHO, 1996). Continued sero-surveillance efforts first isolated DENV-2 in Trinidad in 1953 (WHO, 1996). By 1962, a year before the program ended, 17 of the 47 Pan American Health Organization (PAHO) countries had been certified as *Aedes aegypti* free (WHO, 1996). During the 1960s and 1970s dengue epidemics were few in PAHO countries as a result of the eradication program but once the program ended, countries quickly became re-infested with *Aedes aegypti*. In 1999, Chile was the only country in PAHO that remained free of *Aedes aegypti* (Figure 1) (Arias, 2002). In 1963, DENV-3 was isolated and in 1968 the Americas experienced their first outbreak of DHF in Curacao and Venezuela (WHO, 1996). With isolation of DENV-1 in 1977 and DENV-4 in 1981, the Americas, like Asia, have all four dengue serotypes co-circulating (WHO, 1996).

As described above, the relationship between humans and dengue has been ongoing for at least the last 17 centuries, but not until recently has the overall interaction between species intensified due to expansion of trade, travel, population growth, and urbanization. At present, an estimated 3 billion people are at risk for dengue infection, of which 100 million are estimated to become

infected with the dengue virus annually (WHO/TDR, 2009). This level of risk will lead to approximately 500,000 worldwide cases of DHF and 22,000 deaths (WHO/TDR, 2009; CDC, 2011).

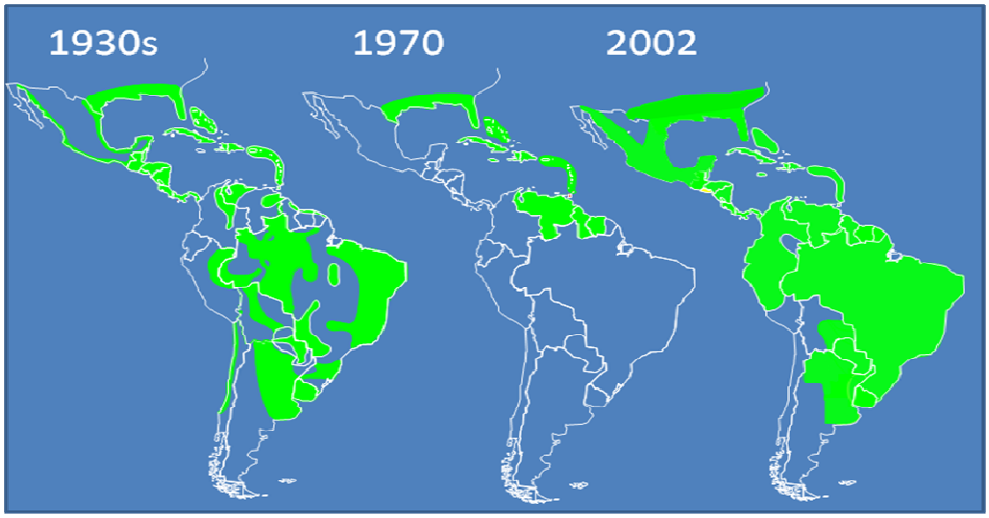


Figure 1. Geographic spread of *Aedes aegypti* in Americas before and after the *Aedes aegypti* eradication program (Arias, 2002). Areas shaded in light green indicate infestation with *Aedes aegypti*.

History of Dengue in Malaysia

The current relationship between Malaysia and dengue can only be fully understood when put in a historical context. During the early 1900's, Malaysia's port cities along the Strait of Malacca rapidly expanded as a result from trade between India, China, and the European naval powers (Saw, 2007). Global

trade brought wealth and influence to Malaysia, but in late 1901, it also brought the first outbreak of dengue fever to the port-city of Penang (Skae, 1902). Following occupation during WWII, dengue outbreaks were observed throughout the country (George, 1987). Exactly when the four different serotypes arrived in Malaysia is unknown; however, dengue had become endemic near the time the first outbreak of DHF was documented in 1962 (George, 1987). Today, Malaysia is endemic for all four distinct dengue serotypes (Vinomarlini *et al.,* 2011).

During the last half century, Malaysia's economy has shifted from primarily agriculture to technology- and service-based industries, which have promoted an estimated rapid increase in urbanization from 20.4% in 1950, to 72.2% in 2010 (UN, 2009b). A rise in urban construction, litter accumulation, and poor drainage increases the number of breeding sites for the diurnal feeding primary urban dengue vector, *Aedes aegypti* (WHO/TDR, 2009). Malaysia, unlike its southern neighbor Singapore, has experienced an increasing cyclic trend in both the number of cases and deaths (Figure 2). The incidence rate of dengue in Malaysia displays an increasing hyperendemic pattern, while dengue is in a cyclic epidemic pattern in Singapore (Figure 3, A). While climates are similar in both countries, a more comprehensive vector population control program, especially with regard to breeding site reduction, in Singapore may aid preventing the hyperendemic pattern of disease observed in Malaysia. Cases-fatality rates have dropped below 1% in both Malaysia and Singapore

demonstrating satisfactory rehydration treatment of dengue patients (Figure 3, B) (WHO/WPR, 2011).

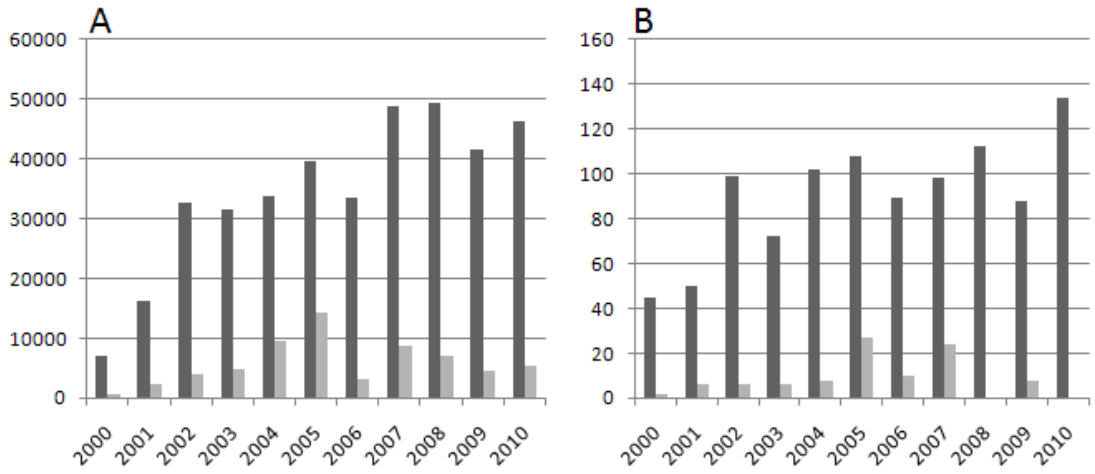


Figure 2. Number of total cases (A) and deaths (B) in Malaysia (black) and Singapore (gray) from 2000 to 2010 (WHO/WPR, 2011).

In a retrospective cross-sectional study from 1998 to 2003, Hussin and colleagues (2005) reported a 1.23 male/female ratio in hospitalized symptomatic dengue infections in Kota Bharu, Malaysia. The pattern of higher rates of reported symptomatic infection in males has been observed in other Southeast Asia Region (SEAR) dengue studies (Yew *et al.*, 2009). Why male hospitalized dengue infections are reported more frequently than female has yet to be fully understood. Potential risk factors for future investigation include increased outdoor activity by males, leading to increased vector exposure, and females

having more coverage of their body with clothing as dictated by religious beliefs, leading to decreased vector exposure.

Studies have observed a protective effect amongst Africans and individuals with African decent (Halstead *et al.,* 2001; Sierra, Kouri, & Guzman, 2007). Researchers have hypothesized genetic polymorphisms within this population maybe the contributing protective factor (Kyle & Harris, 2008). Research on Human Leukocyte Antigen (HLA), a region on human chromosome six that contains immune system genes, may play a major role in development of severe disease. Recent research on Malaysian populations has identified several HLA types to be risk factors for development of severe dengue complications (Appanna, Ponnampalavanar, Lum Chai See, & Sekaran, 2010). The Malaysian population consists of 90% Malay, Chinese and Indian; the remaining 10% is mainly individuals from other SEAR countries and individuals of European descent with very few individuals with African ancestry (J. Sathar, personal communication, March 2011).

In World Health Organization (WHO) SEAR and Western Pacific Region (WPR) member states, children (<15 years) have historically borne the majority of adverse outcomes, in quantity and severity, associated with dengue infection (WHO/TDR, 2009). Research has suggested this maybe in part due to increased capillary fragility among children (Gamble *et al.,* 2000). From 1973, when dengue became a reportable disease in Malaysia, until 1981, the pattern of elevated mortality among children compared to adults persisted. George and

Lam (1997) reported that since 1982 over 50% of dengue related mortality was observed among individuals over the age of 15 in Malaysia. The increased pattern of dengue mortality in adults has also been observed in Thailand and is even more pronounced in Singapore (Cummings *et al.,* 2009). From 2004 to 2008, 24 of the 28 adults who died of dengue in Singapore were older than 45 years of age (Chua, 2010).

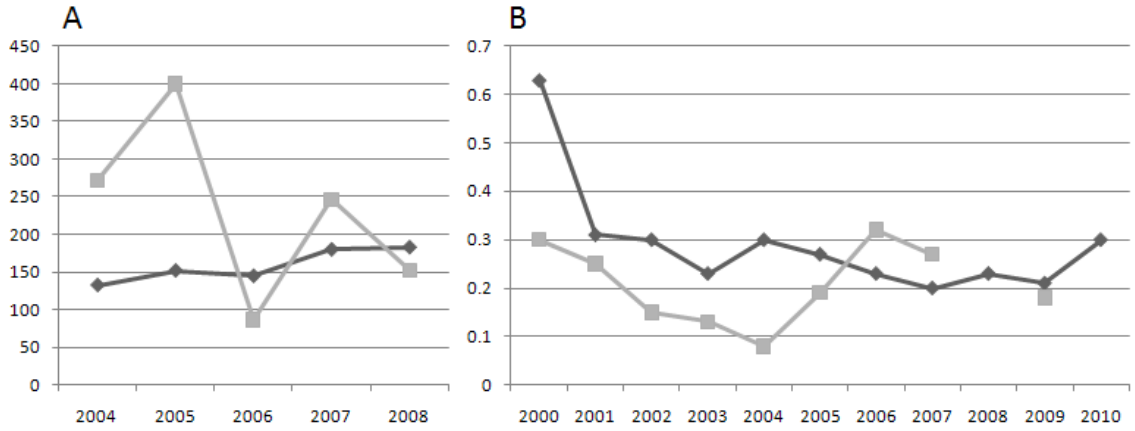


Figure 3. Incidence rate (per 100,000 population) (A) and case-fatality rate (per 100 DF/DHF cases) (B) in Malaysia (black) and Singapore (gray) (WHO/WPR, 2011).

No study to date has undertaken why the average age of dengue mortality has been increasing in Malaysia, but studies have attempted this in other SEAR nations. These studies have suggested the contributing factors to be urban

development, vector control practices, distribution changes in viral serotype and genotypes, change in age distribution, increased immigration and migration patterns, and increasing life expectancy with concurrent decreasing birth rate (Nagao, & Koelle, 2008; Halstead, 1994; Wichmann *et al.,* 2004; Reves, 1985; Cummings *et al.,* 2009). Together these factors may decrease the dengue susceptible population, which reduces the rate that dengue susceptible individuals contract the disease (known as force of infection) (Cummings *et al*., 2009). Consequently, more individuals will contract a secondary heterotypic dengue infection later in life.

Comparing to the above studies' findings, Malaysia already displays many of the contributing trends that may reduce the force of dengue infection. Sustained socioeconomic improvements and a vector control program may decrease the number of people at risk of exposure. Population structural changes also continue to contribute. Since 1970, Malaysia's crude death rate and crude birth rate have been declining, whereas, life expectancy has been increasing (Figure 4) (UNICEF, 2011). These factors will lead to an overall increased median age of the Malaysian population (Figure 5) (UNICEF, 2011), along with increasing the number of non-susceptible dengue individuals further reducing the force of the infection. Additional changes to the Malaysian population structure come as a result of adult immigration from surrounding countries and migration from rural Malaysia where the incidence rate of dengue may not be as high.

The above factors may combine to create longer intervals between dengue infections within the Malaysian population, resulting in a greater proportion of individuals acquiring a secondary infection during adulthood. Observations from Cuba have suggested that increased time between dengue infections increases the risk for severe dengue complications (Guzman *et al.,* 2002).

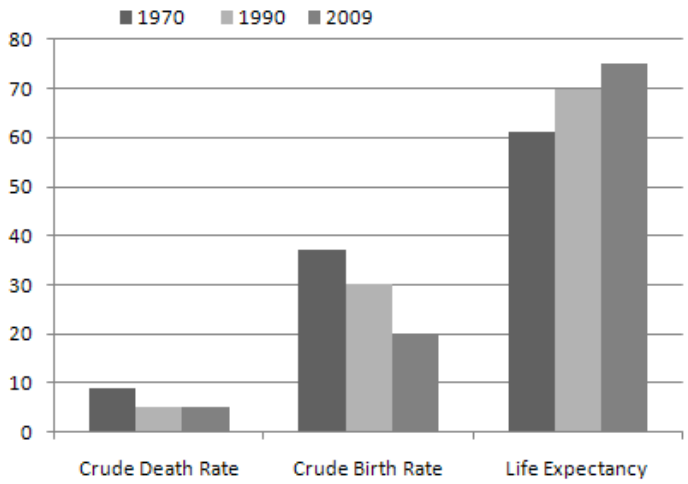


Figure 4. Malaysian crude death rate (per 1000), crude birth rate (per 1000) and life expectancy in 1970, 1990 and 2009 (UNICEF, 2011).

Hypotheses for the biological pathway by which severe dengue complications manifest, include antibody dependent enhancement and reactivation of memory T-cell. Both hypotheses incorporate the notion that a

primary dengue infection alters the immune system, whereby severe complications arise during a secondary heterotypic dengue infection. Integral in the mechanics of both hypotheses are various immune cells such as monocytes, dendritic cells, and T-cells (Halstead, 1994).

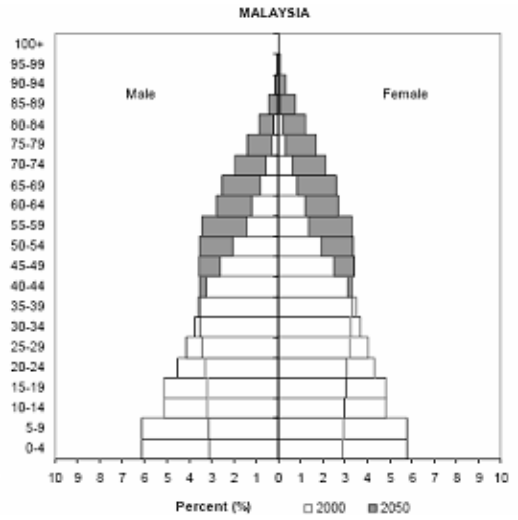


Figure 5. Age-sex structure in Malaysia, 2000 & 2050 (UN, 2009a).

As more countries within Asia, Africa, and Americas enter the second and third phase of Omran's (1971) epidemiological transitions, populations will continue to age, increasing the severe dengue burden upon adults (See Appendix A for discussion of epidemiological transitions). Epidemiological studies in Malaysia to date have described adult dengue manifestations, tried to determine the differences in symptomatic dengue manifestations between adults

and children, and identified potential serum markers in adults as potential biomarkers for early identification of severe dengue cases (Lum, Suaya, Tan, Sah, & Shepard, 2008; Tee *et al*., 2009; Thayan *et al*., 2009). Historically, dengue studies have concentrated on the determinants and biological pathways of severe dengue development within the population aged less than 15 years. With the ongoing shift in burden of severe dengue mortality to individuals above 15 years of age, there is an immediate need to expand investigations on the biological pathway of severe dengue in those above 15 years of age, especially within WHO SEAR & WPR member states.

Statement of the Problem

Estimated to infect upwards of 100 million annually in over 100 nations throughout the pantropics, the equatorial region between the tropic of Cancer and Capricorn, dengue, within the last half century, has emerged as the principal arthropod-borne virus (arbovirus) public health threat. Distribution of global dengue is highly skewed as Southeast Asian and Western Pacific regions endure 75% of the global dengue burden.

Similar to other regional countries, Malaysia has been rapidly urbanizing which has supported a hyperendemic dengue state. A low death rate and high, yet decreasing birth rate has increased the Malaysian population size, median age, and life expectancy. The ongoing epidemiological transition in Malaysia has

potentially raised dengue herd immunity. Consequently, this increases time between dengue infections, which is a risk factor for development of severe dengue, and age at which individuals experience a secondary heterotypic dengue infection. Research has primarily examined severe dengue in children, and extrapolation of findings to adults may only be partial as dengue manifestations have differed between age groups.

The severe dengue biological pathway is not comprehensively understood, although epidemiology and laboratory-based evidence has supported both memory T-cell and antibody independent enhancement hypotheses. Monocytes, pivotal in both hypotheses, have been highly examined through *in vivo* and *in vitro* experimentation (Ubol & Halstead, 2010); however, epidemiological evidence for monocyte involvement is incomplete. Previous dengue infection, determined by the hemagglutinin inhibition assay, is a principal severe dengue risk factor, however, epidemiological assessment of previous dengue infection status with venous absolute monocyte count is lacking.

Uncontrolled capillary effusion, the loss of bodily fluid potentially leading to shock, is the primary cause of mortality from dengue. Monocytes have been indicated as a possible predictor of severe dengue (Potts *et al.,* 2010), although further epidemiological examination is needed to confirm the association taking into consideration independent risk factors.

Significance of the Study

The study is a subset of a data from a larger cross-sectional study that was conducted in a previously un-described dengue population. Dengue manifestations have shown to differ between populations. Description of clinical manifestations during dengue disease of the study population will allow for comparison to the dengue literature. Also, the descriptive undertaking remains important as the current dengue classification criteria was recently updated and there is still a need to ensure the criteria can be effectively used on all global dengue populations.

The study attempted to clarify through epidemiological methods the relationship of venous absolute monocyte count with primary and secondary dengue infection status, taking into consideration the effects of independent risk factors, during the defervescence phase of illness. Monocytes are of particular importance due to their *in vitro* and *in vivo* empirically determined involvement in both innate and adaptive dengue immunity. Among dengue patients, monocytes have been previously observed to decrease in venous blood samples during defervescence. As a previous dengue infection has been observed to be a significant risk factor for severe dengue complications during a heterotypic secondary dengue infection, determining if previous dengue infection status alters immunological responses, specifically venous absolute monocyte count, is of significant interest in elucidating the severe dengue biological pathway. Understanding how independent risk factors affect the relationship between

primary and secondary dengue infection and monocyte level, may provide currently absent epidemiological support and clarity on the antibody dependent enhancement and/or reactivation of memory T-cell severe dengue hypotheses.

Additional epidemiological assessment of the association between monocytes and the outcome of vascular leakage/hemorrhage, while considering independent risk factors, may provide further insights into the severe dengue biological pathway with respect to monocyte involvement.

Purpose of the Study

The primary purpose of this cross-sectional study was to collect data and analyze the association of venous absolute monocyte count with primary and secondary dengue infection status during defervescence considering the role of independent risk factors (e.g. gender, age, race, and distance from patient residence to hospital) among hospital admitted dengue patients above 15 years of age in Federal Hospital Ampang, Selangor, Malaysia from June to December 2010.

Aims of the Study

Primary aims of the study include:

1) Describe the demographics, co-morbidities, manifestations, and laboratory results of the study population by primary & secondary dengue infection status and absence & presence of hemorrhage/vascular leakage.

2) Determine the relationship of venous absolute monocyte count with primary and secondary infection considering the effects of the independent risk factors gender, age, race, and distance of residence from hospital, during defervescence.

Secondary aims of the study include:

1) Determine the relationship of venous absolute monocyte count with presence and absence of hemorrhage/vascular leakage, considering the effects of the independent risk factors gender, age, race, and distance of residence from hospital, during defervescence.

LITERATURE REVIEW

A literature review was performed using the following searchable published literature databases: PubMed, Web of Knowledge and Science Direct. The searches used the keywords: dengue, monocyte, macrophage, secondary infection, hemorrhage, and vascular leakage. Results were limited to English written articles and were extracted by March 25th, 2011.

The Unknown Severe Dengue Biological Pathway

A literature review on the development of severe dengue reveals multiple hypotheses all of which have biological plausibility (Rodenhuis-Zybert, Wilschut, & Smit, 2010). The elusiveness of conclusive empirical evidence pointing toward a single hypothesis has prompted experts to suggest that the ultimate causal pathway for the development of severe dengue complications is most likely multifactorial (Whitehorn & Farrar, 2010). Central hypotheses for the development of severe dengue include: viral virulence, reactivation of memory T-cells, HLA type, and antibody-dependent enhancement (ADE) (Kyle & Harris, 2008). As the study is epidemiological in nature, the intended purpose of the below section is to not present a comprehensive review of the microbiology of

dengue pathogenesis; dengue pathogenesis reviews may be found elsewhere (Ubol & Halstead, 2010; Rothman, 2010; Kyle & Harris, 2008). The intended purpose is to introduce a generalized platform of knowledge which allows for understanding of the current literature gap and interpretation of study results.

The hypothesis of viral virulence revolves around the central idea that a particular serotype or genotype of dengue virus is inherently more prone to cause severe dengue complications (Whitehorn & Farrar, 2010). In 1997, an outbreak of DENV-2 was detected in Cuba which produced high incidence rates of severe dengue manifestations (Guzman, Kouri, & Halstead, 2000). Genotyping of the 1997 Cuban virus indicated mutations had occurred compared to a similar yet less severe Jamaican DENV-2 strain (Guzman *et al.,* 2000). Under the viral virulence hypothesis, a mutation in the 1997 Cuban DENV-2 strain increased the virulence of the virus. The viral virulence hypothesis alone does not support observed epidemiological evidence as many people will be infected with dengue during an epidemic, however, only a fraction of cases will develop severe complications (Martina, Koraka, &Osterhaus, 1999).

Intrinsic risk factors, especially the involvement of the host immune response, provide a more promising explanation for why only a small proportion of dengue infected individuals develop severe dengue complications. As previously explained, studies have identified associations between HLA type and dengue disease severity (Appanna *et al.*, 2010). Further research on HLA types have the potential to add knowledge to the pathogenesis theories of dengue, and

can also be used as a tool to identify individuals at an increased risk for dengue disease (Kyle & Harris, 2008). Larger studies are needed to confirm and expand initial findings.

The reactivation of memory T-cells and ADE hypotheses are both based on the notion that following a primary infection, the host immune system is altered or primed such that upon a secondary infection, a severe immunological reaction occurs (Pang, Cardosa, & Guzman, *2007*). Central to both hypotheses are the involvement of monocytes. Produced from progenitor cells in the bone marrow and stored in the spleen, monocytes are the parent cells found naturally in the circulatory system that undergo differentiation and activation into macrophages and dendritic cells once they enter tissues (Imhof & Aurrand-Lions, 2004) (Figure 6).

Antibody dependent enhancement in dengue is supported by the epidemiologic findings of increased risk during a secondary heterotypic dengue infection (Ubol & Halstead, 2010). During a primary dengue infection the adaptive immunity pathway is activated and B-cells produce antibodies specific against the infecting serotype. However, antibodies which are cross-reactive with the other dengue serotypes are also produced (Pang *et al.,* 2007). The cross-reactive antibodies provide a transient period of time that the individual is immune to all dengue serotypes. After roughly three months the cross-reactive antibodies are no longer maintained at levels capable of providing complete dengue protection (Dengue Virus Net, 2011). The primary dengue infection has

altered the immune system in that cross-reactive antibodies are present, but at non-neutralizing levels (Kyle & Harris, 2008).

During a heterotypic secondary dengue infection, cross-reactive immunoglobulins from a primary dengue infection bind to the virus of the secondary infection. As cross reactive antibodies have dropped below neutralizing levels, inactivation of the dengue virus is not achieved. Instead, the dengue virus and cross-reactive antibody attach to macrophages/monocytes via their Fc-receptor (Pang *et al.,* 2007). Dengue virus has been shown to be capable of infecting dendritic cells, monocytes, macrophages, lymphocytes, hepatocytes and vascular endothelial cells (Leong, Wong, Leong, Tan, & Wannakrairot, 2007). Replication of dengue virus has been observed in dendritic cells, but primarily occurs in monocytes & macrophages (Rodenhuis-Zybert *et al.,* 2010).

Halstead and colleagues (2010) have suggested an intrinsic ADE model in which binding via the Fc-receptor on a monocytic cell of a cross-reactive antibody and dengue virus enhances dengue viral replication within the monocytic cell followed by proinflammatory cytokine output (Halstead, Mahalingam, Marovich, Ubol, & Mosser, 2010) (Figure 7). The intrinsic ADE model is supported by epidemiological evidence in which high levels of viremia and proinflammatory cytokines are associated with increased risk of severe dengue complications (Kyle & Harris, 2008; Pang *et al.,* 2007; Avirutnan *et al.,* 2006; Endy *et al.*, 2004; Libraty *et al.,* 2002). The ADE hypothesis can also explain the appearance of

severe dengue in infants. Pengsaa and colleagues (2003) reported that maternally transferred dengue neutralizing antibodies were detectable at decreasing levels in infants until nine months after birth. Under the ADE hypothesis, an infant with a primary dengue infection and non-neutralizing levels of maternal anti-dengue antibodies would be at increased risk for developing severe dengue complications.

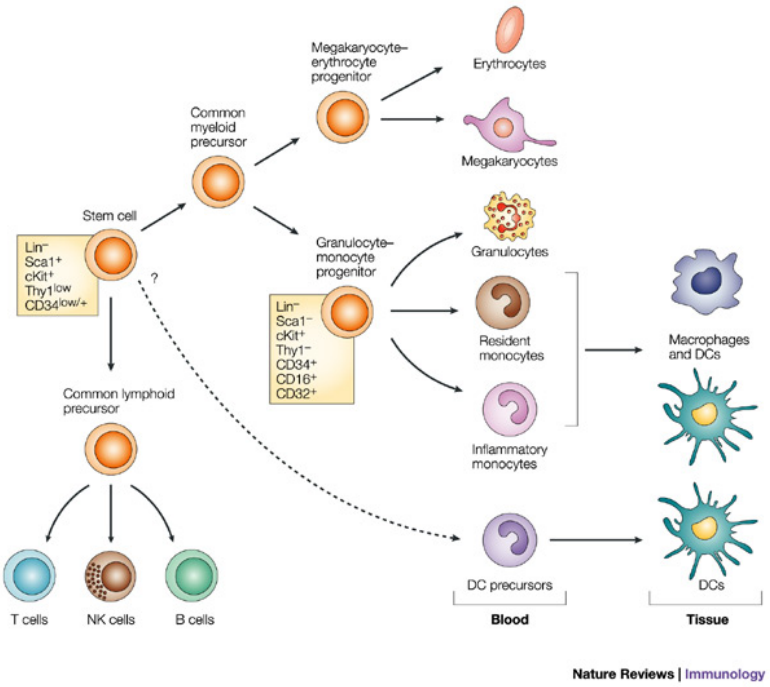


Figure 6. "Origin and haematopoietic differentiation of myeloid antigen-presenting cells" (Imhof & Aurrand-Lions, 2004).

The ADE hypothesis is supported by the epidemiological finding that secondary dengue infection is a risk factor for development of severe dengue;

however, key holes in the dengue ADE literature fail to explain all epidemiological evidence. Severe dengue complications have been observed to manifest during the defervescence phase of dengue illness, which approximately occurs on day five of symptomatic illness (WHO/TDR, 2009). ADE incorporates innate immunity, which has a rapid response time to infection. As currently presented, dengue ADE does not explain the epidemiological evidence of delayed manifestations of severe dengue complications as viremia levels have been observed to decrease significantly by the onset of defervescence (WHO/TDR, 2009).

Under the reactivation of memory T-cells hypothesis, during a primary dengue infection the virus is phagocytized by macrophages/monocytes and viral antigens are presented on the cell membrane to activate memory T-cell production (Pang *et al.,* 2007). As the four dengue serotypes have 60-70% homology, cross-reactive memory T-cells will potentially be produced (Wong *et al.*, 2007). During a secondary heterotypic dengue infection, the virus will again be phagocytized by macrophages/monocytes and viral antigens displayed on the surface. Cross-reactive memory T-cells produced during the primary infection recognize the viral antigens displayed by macrophages/monocytes during the secondary heterotypic dengue infection, which results in the activation of the memory T-cell and release of cytokines (Beaumier, Mathew, Bashyam, & Rothman, 2008). Memory T-cell released cytokines have been observed to attract immunologic cells, such as: macrophages, monocytes and memory T-

cells to the site of infection, subsequently increasing the level of cytokine release (King, Anderson, & Marshall, 2002).

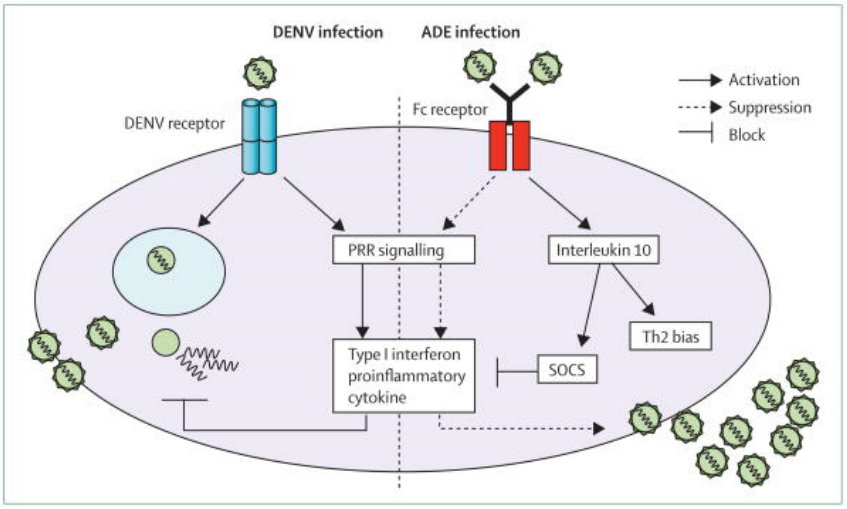


Figure 7. "Intrinsic antibody-dependent enhancement of dengue virus infection in THP-1 human monocytic cells." ADE=antibody-dependent enhancement. Th2=Thelper 2. DENV=dengue virus (Halstead *et al.,* 2010).

Raised levels of activation and proliferation of memory T-cells in dengue infection has been associated with increased risk for severe dengue complications (Rothman, 2010). Among the cytokines released by the memory T-cells are proinflammatory cytokines (i.e. interferon gamma & tumor necrosis factor alpha) (Pang *et al.,* 2007; Rothman & Ennis, 1999). Elevated levels of proinflammatory cytokines have been associated with increased vascular

permeability during dengue infection (Mongkolsapaya *et al.,* 2006). Vascular leakage is the primary mechanism for dengue infection resulting in mortality (Avirutnan *et al.,* 2006). As memory T-cell attraction and activation at the site of infection is a slower immunological process than the innate immune response hypothesized in ADE, the reactivation of memory T-cells hypothesis can better explain the epidemiological observation of delayed severe dengue complication manifestation (Nielsen, 2009).

While heavily investigated with *in vitro* and *in vivo* studies due to their role in both ADE and memory T-cell hypotheses, monocytes have remained poorly examined under epidemiological studies. A literature review on monocytes/macrophages investigated under epidemiological studies has revealed the following. In children (<15 years of age), clinical epidemiological studies have observed significantly lower absolute monocytes counts in dengue patients compared to other febrile illnesses (Kalayanarooj *et al.,* 1997; Green *et al.,* 1999). Monocyte activation has been associated with severe dengue manifestations (Durbin *et al.,* 2008). Potts and colleagues (2010) have used classification and regression tree analysis to identify in Thai pediatric patients clinical laboratory predictor models for severe dengue. Their results found that a percent monocyte cut-off of ≤ 9%, along with other variables, was capable of identifying patients with dengue shock syndrome (Potts *et al.,* 2010). An epidemiological study in adults found an association by dengue serotype of higher monocyte count in DENV-2 compared to DENV-1 (Tsai *et al.,* 2009).

The literature review undertaken did not return an epidemiological study which investigated the relationship of venous absolute monocyte count with primary and secondary dengue infection status or relationship of venous absolute monocytes with absence or presence of hemorrhage/vascular leakage. Therefore, the study described here attempts to fill the literature gap of absolute monocyte count in primary versus secondary dengue infection during the defervescence phase of illness. The study also attempts to assess the use of absolute monocyte count as an indicator of severe manifestations using novel outcome criteria.

The Epidemiological Triad

The epidemiological triad is a traditional model for infectious disease causation comprised of the components external agent, susceptible host, and an environment, which brings the agent and host in contact. To model the complex relationships found in vector-borne diseases, the traditional infectious disease epidemiological triad has been supplemented to include a centralized vector component (Chadee, Williams, & Kitron, 2005). The transmission cycle of an infectious disease can be interrupted with reduction or removal of a single model component; however, the presence of all model components in sufficient quantities can result in epidemics.

During the past 60 years, global components of the dengue epidemiological triad have all dramatically increased resulting in a significant rise in dengue incidence worldwide (Guha-Sapir & Schimmer, 2005) (Figure 8). In the following section, the dengue epidemiological triad of disease causation is discussed by each contributing factor providing a background on the changing interactions.

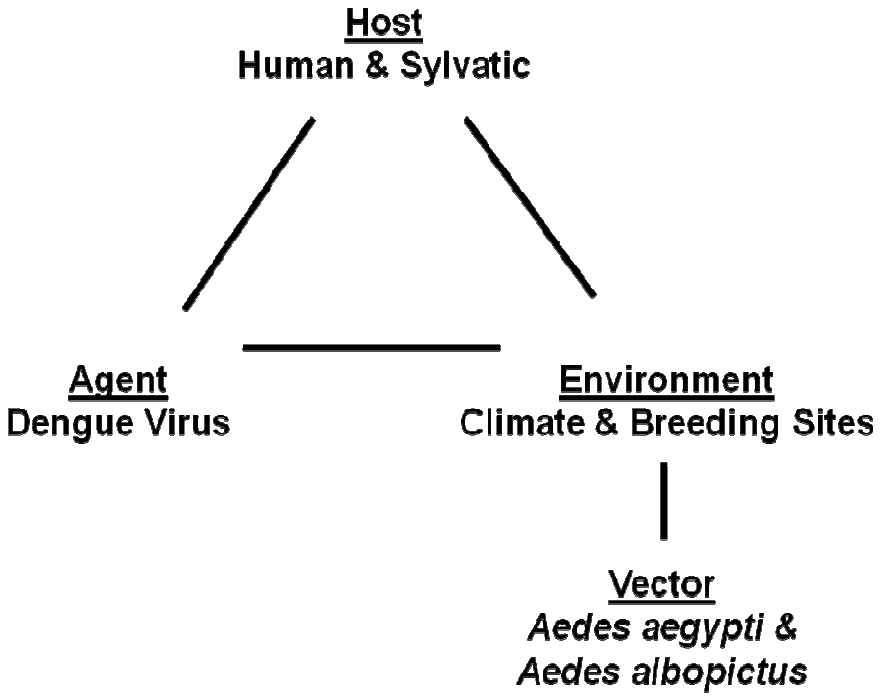


Figure 8. Dengue epidemiologic triad of disease causation (PSU, 2010) edited.

Agent

Dengue virus is a member of the large pathogenic virus family *Flaviviridae* that includes more than 70 other medically important tropical diseases, including yellow fever, Japanese encephalitis, hepatitis C, and West Nile virus (Lupi, 2011) (Figure 9). Within the virus genus *Flavivirus*, dengue virus is the culpable agent responsible for the highest human case-fatality rate (Qi, Zhang, & Chi, 2008). The mapped 11kb genome of the enveloped positive single stranded RNA dengue virus encodes for a viral particle composed of three structural and seven nonstructural proteins (Perera, & Khun, 2008). There are four serologically similar dengue viruses (DENV-1, -2, -3, and -4) each with three to five genetic groups (genotypes). Each serotype of dengue is genetically and anti-genetically diverse (Perera, & Khun, 2008).

Vector

Dengue is transmitted by the mosquito-vectors *Aedes aegypti* and *Aedes albopictus*. Unlike the malaria vectors of the genus *Anopheles*, the dengue vectors are active during daylight hours with peak feeding activities occurring during at dawn and dusk (Jansen & Beebe, 2009). Adult *Aedes* females favor human blood meals and are capable of biting multiple hosts in a single gonotrophic cycle (Jansen & Beebe, 2009). Preferred breeding sites of *Ae. albopictus* are small natural or man-made stagnant fresh water containers in rural

or peri-urban areas (Paupy, Delatte, Bagny, Corbel, & Fontenille, 2009). On the other hand, *Ae. aegypti* is found primarily in fast growing urban settings that allow for innumerable man-made breeding locations (Jansen & Beebe, 2009). *Aedes aegypti* is an adaptive vector fully capable of sustaining a reproducing population within human dwellings (Jansen & Beebe, 2009).

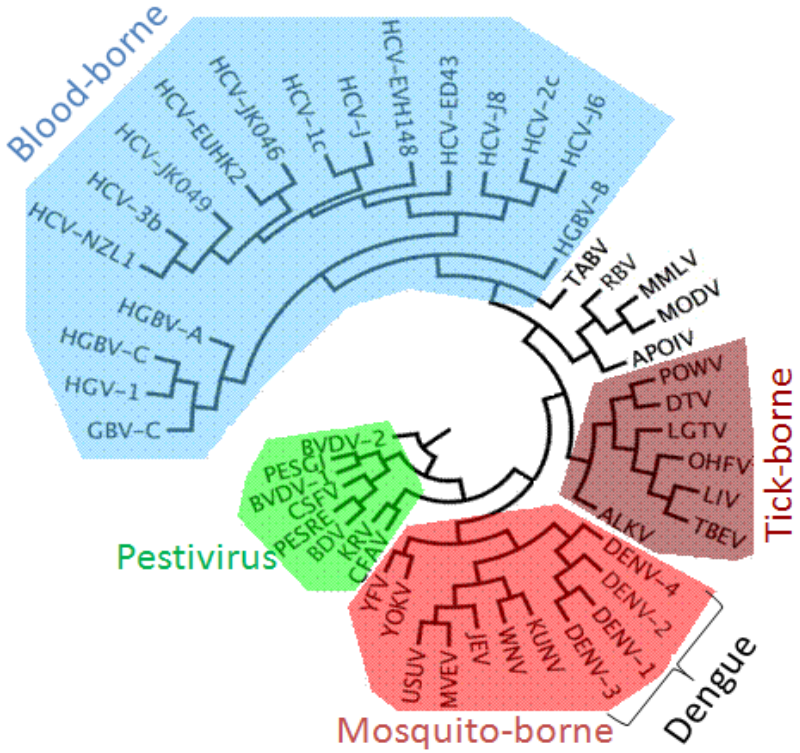


Figure 9. "*Flaviviridae* phylogenetic tree (unrooted)" (UTMB, 2009) edited.

Life Cycle

The *Aedes* vectors have a four stage lifecycle: egg, larvae, pupae, and adult (Figure 10). The eggs of *Aedes* are highly durable surviving in desiccated states for over a year and remaining viable after wintering in frozen water. Eggs must be laid in fresh water and hatch within 2-7 days when temperatures are greater than 12℃. The development and survival of larvae is dependent on environmental conditions, including temperature, humidity, predation, and water depth (>10mm). Larvae pupate after a minimum of 4 days after reaching a minimum weight. The pupae stage lasts for 2 days in temperatures greater than 18℃ from which the adult mosquito emerges. The avera ge lifespan of an adult Aedes is two weeks (CDC, 2011; Jansen & Beebe, 2009).

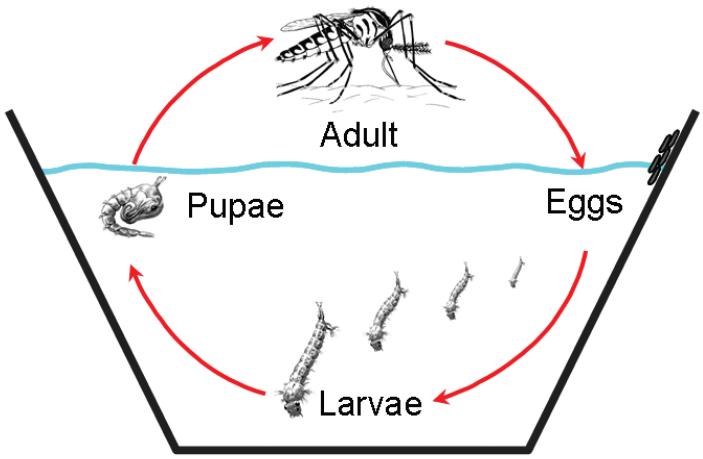


Figure 10. Lifecycle of *Aedes aegypti & Aedes albopictus* (CDC, 2011).

Natural History

The dengue virus lifecycle is human-mosquito-human. Dengue is also transmitted in a non-human primate-mosquito-non-human primate lifecycle (Kyle & Harris, 2008). These sylvatic cycles have been demonstrated in both Asia and Africa (Gubler, 1998).

The adult female *Aedes* mosquito, after taking a blood meal on a dengue viraemic host, has an extrinsic viral incubation period of 8-10 days. The extrinsic incubation period refers to the time it takes for the dengue virus to replicate in the abdomen and disseminate into the mosquito's salivary glands (Salazar, Richardson, Sánchez-Vargas, Olson, & Beaty, 2007). During feeding, the mosquito injects saliva into the host that contains proteins to interrupt the host clotting cascade to allow the mosquito to take a blood meal. The mosquito will remain infectious for the remainder of the normal one to two week *Aedes* lifespan, even after multiple feedings. The human intrinsic incubation period lasts for 3-14 days, with an average of 4-7 days, after which viremia levels are elevated enough that a mosquito taking a blood meal can acquire the dengue virus (CDC, 2011). Vertical transmission of dengue within vectors has been demonstrated (Thenmozhi *et al.,* 2007).

Environment

Climate

The dengue vector lifecycle (adult survival, reproduction rate, and egg, larvae, and pupae development) is highly influenced by climatic factors, including temperature, precipitation, and humidity. Tropical conditions with high temperature, humidity levels, and precipitation are ideal conditions that promote elevated mosquito populations and rapid extrinsic incubation periods. A shortened extrinsic period increases the risk for dengue infection to the human population as adult mosquitoes will be infectious for a longer duration of their lifespan (CDC, 2011).

Due the reproductive nature of the dengue vectors, a cyclical pattern in dengue disease is observed in response to the monsoon season. During the monsoons, breeding sites, both natural and man-made, become more abundant allowing for a spike in the mosquito population, subsequently increasing the risk for dengue infection. This cyclic pattern of peaking dengue cases during the summer monsoon months can be observed in Figure 11, depicting the number of reported dengue cases in Thailand per month from 1990-2007. Dengue vectors' inherent propensity to thrive under tropical climatic conditions has concentrated the risk of dengue around the global equatorial belt; however, historic dengue surveillance has observed the worldwide distribution of dengue to not simply follow climatic conditions.

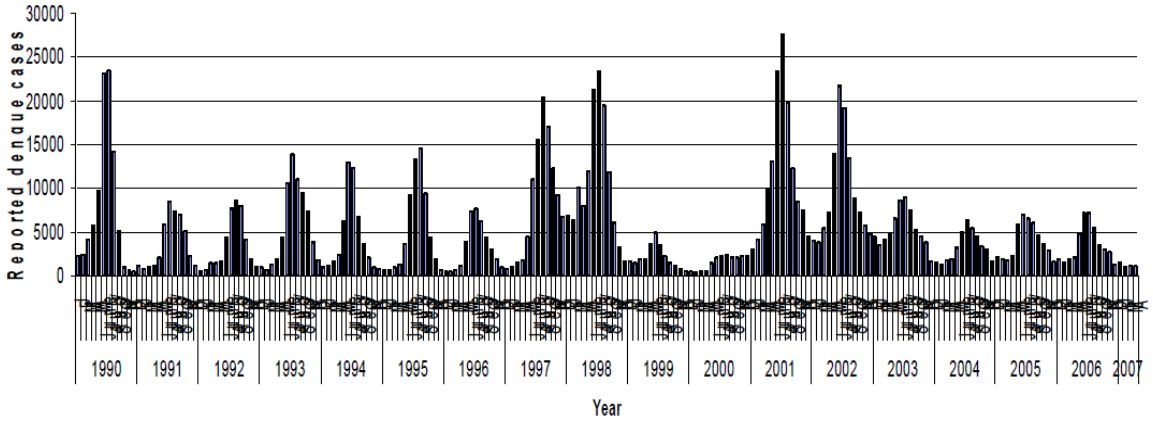


Figure 11. Thailand: Seasonal trend of dengue cases (1990-2007 April) (WHO/SEAR, 2011).

Surveillance

Dengue surveillance is an extremely beneficial practice that has elucidated temporal and spatial trends on global, regional, and local levels. Understanding the general trend of dengue over time has been achieved by graphing the total number of cases with dengue and countries reporting dengue cases to WHO from 1955 to 2005 (Figure 12) (WHO/TDR, 2007). The number of countries reporting dengue cases jumped in the early 1960s, followed by a plateau until the early 1980s. Since the early 1980s, countries reporting dengue cases have been gradually increasing. The total number of cases did not begin to rise significantly until the late 1970s. A cyclic increasing trend is noticeable in the number of countries reporting dengue cases and the total number of cases reported; however, their patterns do not mirror each other. A definitive answer for why dengue total cases fluctuate with year is complex and geographically-

specific, as there are many contributing factors (see Extrinsic Risk Factors) (Kyle & Harris, 2008).

To investigate the trend of dengue at the country level, the incidence rates of reported dengue fever (DF)/DHF per 100,000 in selected WHO SEAR member states was graphed (Figure 13). Each member state presents a cyclical pattern in their incidence rate, though the rate of that pattern varies within each state and between states. Variation within dengue surveillance among geographically close countries allows for the inference that risk factors that operate within countries heavily influence the incidence rate of dengue.

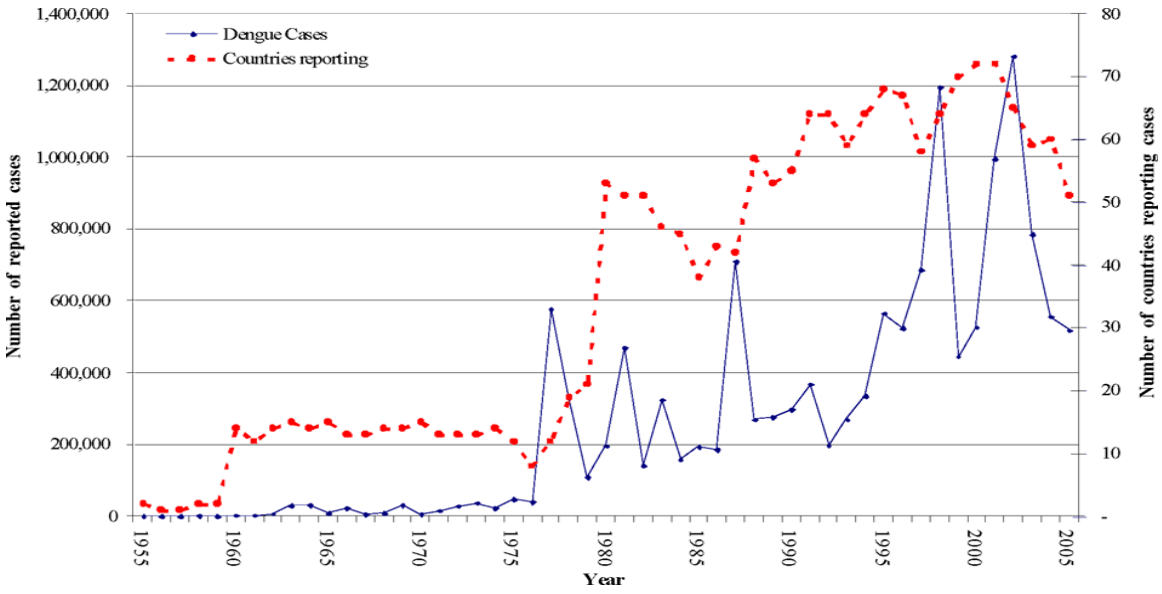


Figure 12. Dengue cases: Global annual number of cases reported and number of countries reporting to WHO by year, 1955 to 2005 (WHO/TDR, 2007).

Serotype-surveillance of dengue infections allowed for the discovery that typically only one or two serotypes are predominant for a period of one-three years (Figure 14) (Adams, *et al.,* 2006). This information has permitted insight on how dengue can maintain high incidence overtime. A population experiencing high transmission of a serotype(s) for a period of time will result in a decrease of overall susceptibility within the effected population to that serotype(s). The drop in transmission of the dominant serotype(s) will allow a different serotype, which has higher levels of susceptibility in the population, to emerge. This relationship permits the serotypes to change, but will keep the incidence rate at elevated levels.

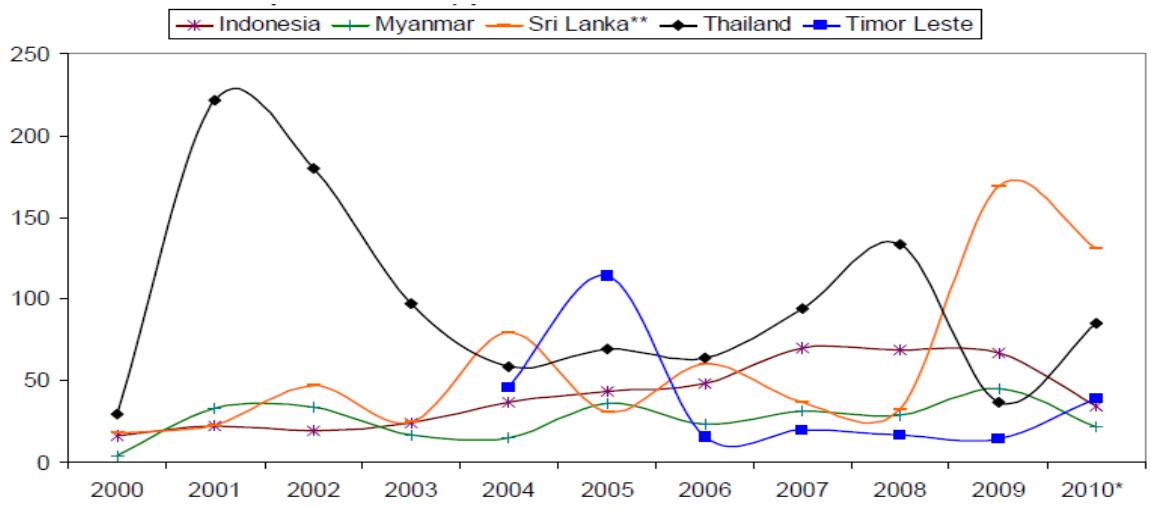


Figure 13. Incidence rates of reported dengue fever & dengue hemorrhagic fever per 100,000 in selected World Health Organization Southeast Asia Region member states (WHO/SEAR, 2011).

Total DHF cases reported to WHO have been graphed by year for both the Americas and Asia (Figures 15 & 16) (Arias, 2002). Both regions display an increasing cyclic trend, although the Americas have fewer overall reported DHF cases. As previously discussed, the lag time in the appearance of DHF in the Americas was primarily due to the *Aedes aegypti* eradication program run from 1947 to 1963. Overlaying the total reported DHF cases of the Americas and Asia by year starting for each at the first recorded observance of DHF presents a striking similar pattern (Figure 17) (Arias, 2002). If risk factors of dengue continue to exacerbate the situation in the Americas, one can make an inference that DHF may approach that currently observed in Asia, after controlling for population differences.

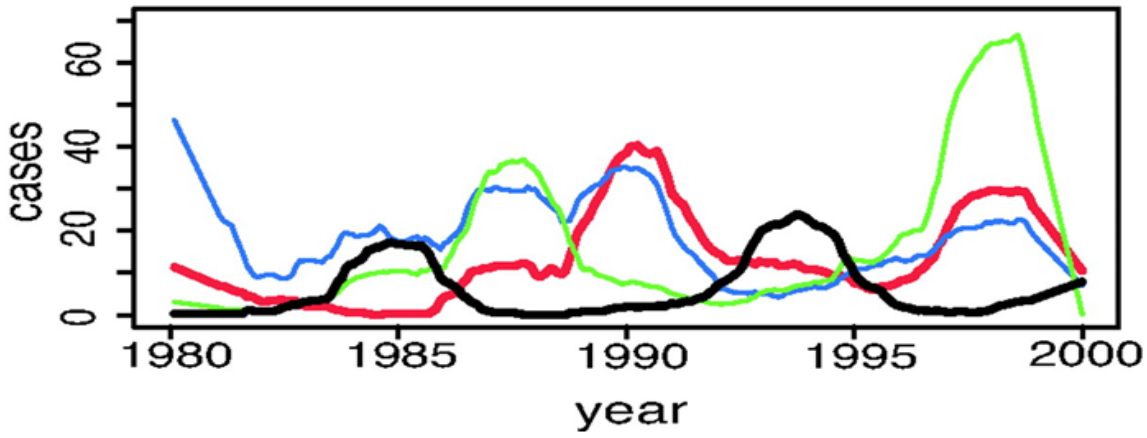


Figure 14. "Monthly confirmed cases of dengue by serotype at Queen Sirikit National Institute of Child Health, Bangkok 1980 to 2000" Red, DENV-1; blue, DENV-2; green, DENV-3; black, DENV-4 (Adams, *et al.,* 2006).

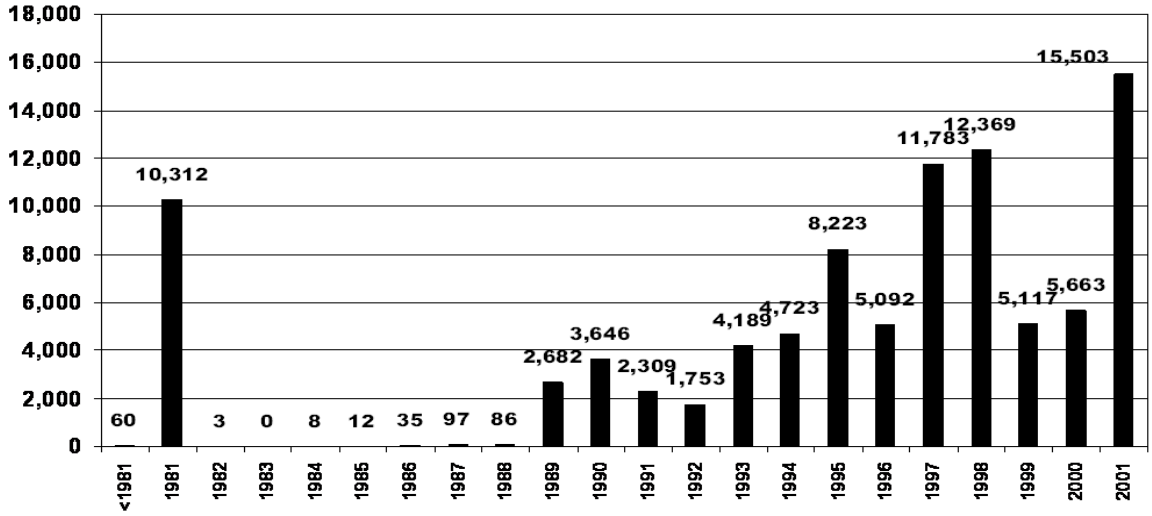


Figure 15. Total reported cases of dengue hemorrhagic fever in the Americas by year from 1981-2001 (Arias, 2002).

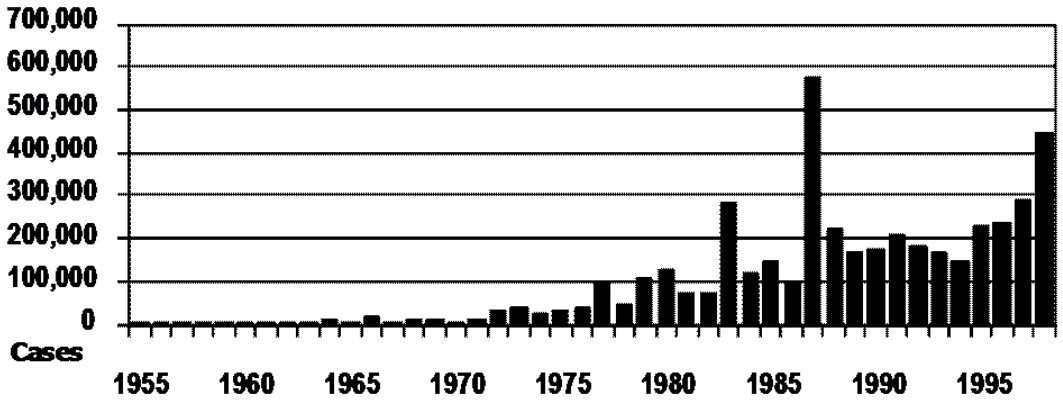


Figure 16. Total reported cases of dengue hemorrhagic fever in the Asia by year from 1955-1999 (Arias, 2002).

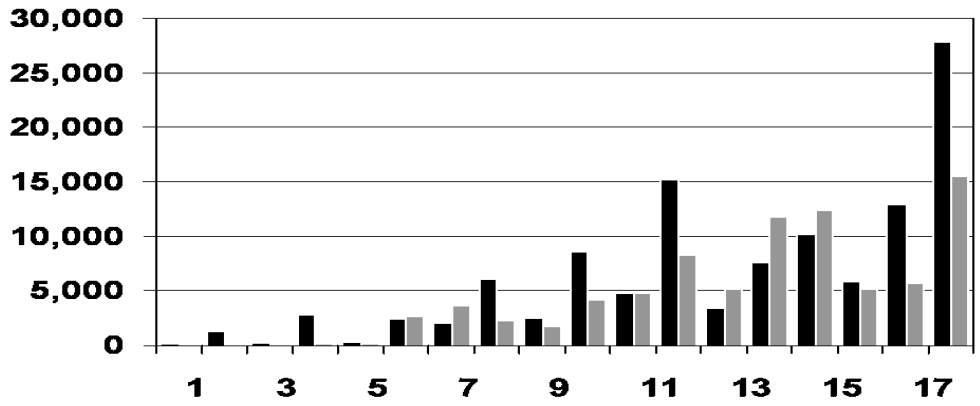


Figure 17. Total reported cases of dengue hemorrhagic fever in the Asia and Americas by year matched by first year of appearance (Arias, 2002).

Plotting the dengue case fatality percent for DHF of the member states of the WHO Southeast Asia region by year from 1985 to 2006 demonstrates a clear cyclic decreasing trend (Figure 18) (WHO/SEAR, 2011). The cyclic trend might be influenced by many risk factors, including viral strain, intrinsic factor (i.e., genetics), susceptible population, prevention practices, and climate factors. Reasons as to why some years see greater case fatality among DHF patients has yet to be fully deduced. The overall decreasing trend observed in the case fatality percent since 1985, may in part be due to greater awareness of the disease among medical personnel and proper management of rehydration therapy. Under proper medical care, DHF patients typically experience a case fatality rate of around 1% throughout the world (Kyle, & Harris, 2008). Although

with absent or improper care, case fatality rates have been observed in DHF patients as high as 20% (Monath, 2007).

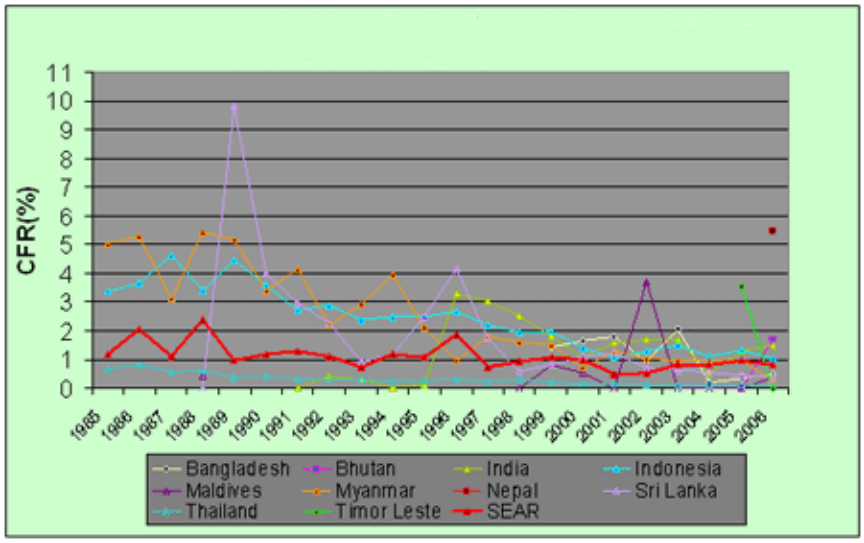


Figure 18. Case fatality rate of countries within the World Health Organization Southeast Asia region (WHO/SEAR, 2011).

Extrinsic Risk Factors

Extrinsic risk factors for dengue are associated with the environment, and influence the amount of risk for acquiring a dengue virus infection at a particular location. Recognized extrinsic risk factors for dengue include endemic dengue vector population, mass transportation, water access, urbanization, population growth, waste accumulation, dengue susceptibility of the population, climate,

circulating dengue viral strain, housing type, population density, and preventative measures implemented in the community (Gubler, 1997).

World Health Organization marks the 10℃ July and Janua ry isotherms as the year-round boundary of risk for dengue infection (Figure 19) (WHO, 2010). Concerns have been raised as to whether global warming will contribute to the geographic spread of dengue risk. Researchers have modeled the hypothetical increase in risk from dengue with global temperature rise (Figure 20) (Hales, de Wet, Maindonald, & Woodward, 2002).

Jansen and Beebe (2010) argue that global models based solely on climatic factors do not accurately model increased risk for dengue. They present the example of southern Australia where climatic shifts have reduced rainfall totals significantly below historical levels. A climatic model would indicate the region to be undergoing a reduction in risk for dengue, but the authors point the human element. As southern Australia dries, the government and private citizens have created more water retention structures (i.e. dams, cattle troughs, rain barrels, etc.). *Aedes* is highly adapted to human environmental intervention which in southern Australia may lead to an overall increased dengue risk if proper preventative steps to reduce breeding sites are not taken.

Figure 19. Countries reporting dengue in 2010 (WHO, 2010).

The extrinsic risk factors that have and continue to hold the most potential for influencing the epidemiology of dengue are mass transportation, population growth, and urbanization. The last half century has seen drastic changes in the global distribution of the dengue vectors (Guzman & Istúriz, 2010). Mosquitoes have very limited self dispersion capabilities (Russell, Webb, Williams, & Richie 2005), making the vast geographic spread of the dengue vectors attributed to increased international commerce and mass mobilization of the human population (Kyle & Harris, 2008). In 2006, an estimated 2.1 billion individuals around the world boarded a plane (Mangill & Gendreau, 2005), while last year, there were 935 million international tourists (WTO, 2011). As the human

population continues to grow in mobility, so do the chances that an infectious disease will breakout into a new geographic location (Armelagos, 1998).

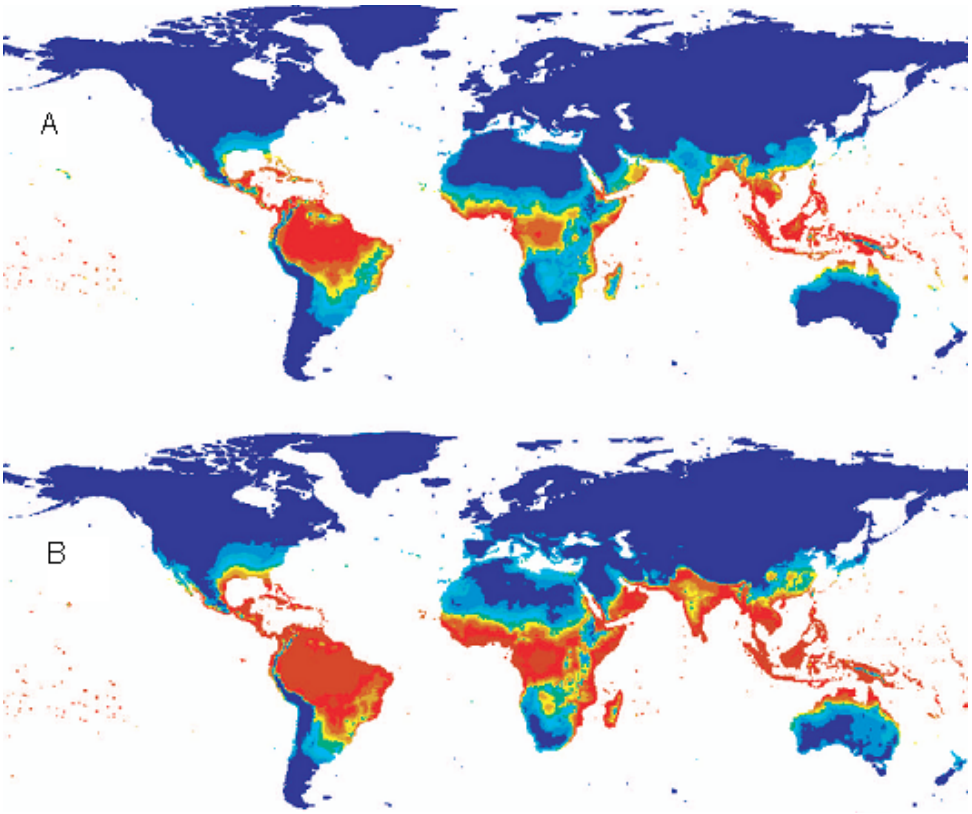


Figure 20. "Estimated baseline population at risk in 1990 (A) and estimated population at risk in 2085 (B)" Range: Dark Blue 0.0-0.1 to Red 0.9-1.0 (Hales *et al*, 2002).

Since peaking in the mid-1960s, global population growth rates have been steadily declining (Figure 21); however, the small decreases in growth rate are

not enough to decrease the overall global population within the next 40 years (Figure 22) (US Census Bureau, 2011). Continued growth of the world population will raise the risk for dengue infection as there will be more opportunities for transmission with dengue susceptible individuals.

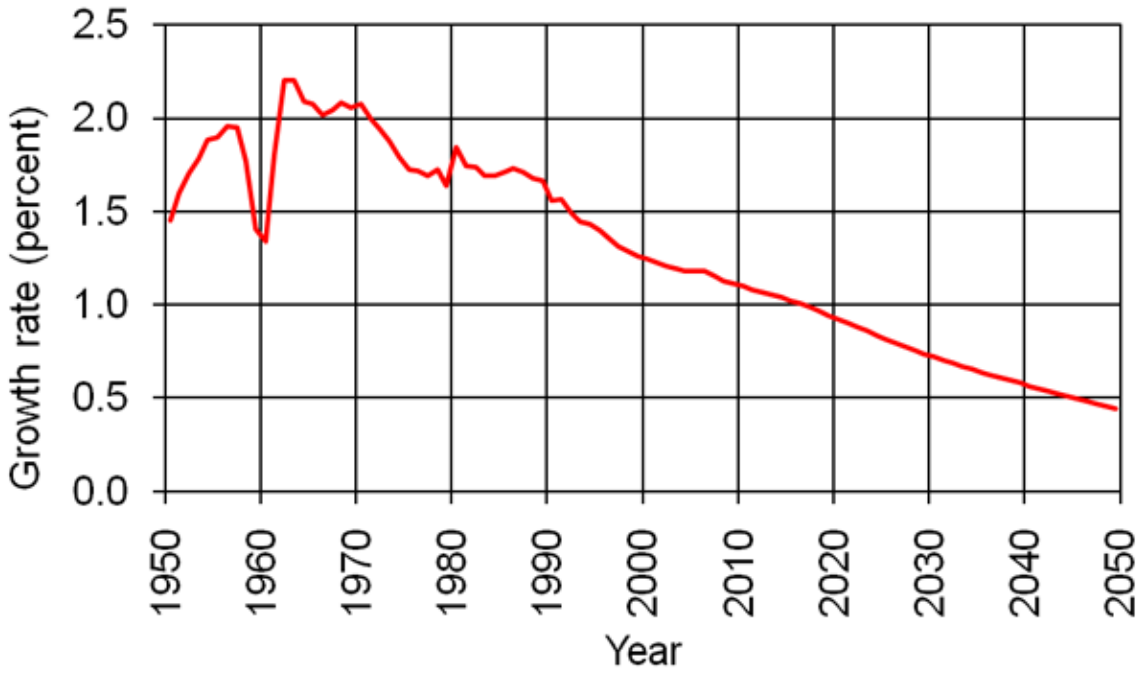


Figure 21. World population growth rate: 1950-2050 (US Census Bureau, 2011).

Urbanization is a significant extrinsic risk factor for dengue infection (Kyle, & Harris, 2008). The minimum population necessary to sustain dengue transmission is estimated between 10,000 to 1,000,000 individuals (Gubler, 1997). In these populations, there are enough dengue susceptible individuals

that come in contact with the infected vectors to allow for continued disease transmission. In less developed nations, population growth may outstrip infrastructure for proper housing, sanitation, and potable water. As a result, increase fresh water filled containers in and around human residences may occur. These ideal breeding sites for dengue vectors will allow for greater vector populations within urban environments (Kyle, & Harris, 2008).

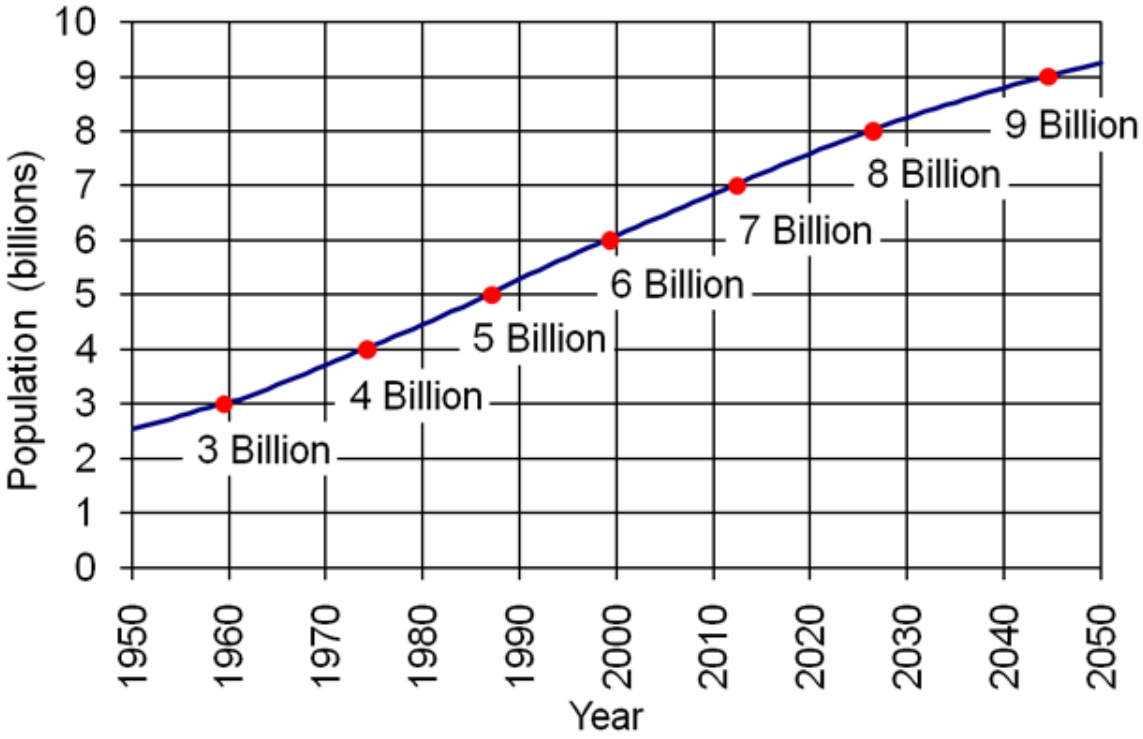


Figure 22. "World Population: 1950-2050" (US Census Bureau, 2011).

The presence of multiple co-circulating dengue serotypes is a significant risk factor for the development of severe dengue. Geographic distribution of dengue serotypes is uneven and should be taken into consideration during severe dengue predictive modeling. Fried and colleagues (2010) found no statistically significant difference in development of dengue hemorrhagic fever by serotype.

The relationship between the dengue and the environment is highly complex and cannot be modeled globally solely using climatic indicators. With proper consideration to all extrinsic risk factors including "urbanization, socioeconomic factors, building design, water supply and management, and public health services," Jansen and Beebe (2010, p.274) suggest that accurate modeling maybe possible at the local and regional levels.

Global Burden of Dengue

Currently, dengue has a global reach including developed and less developed nations; however, the burden of dengue is geographically highly stratified. Today, the WHO SEAR and WPR contain 75% of the global of the global dengue burden (WHO/SEAR, 2011). In the WPR, 25 of 37 states and territories reported dengue cases; whereas the Democratic Peoples Republic of Korea was the only SEAR dengue member state not reporting native transmission of dengue (WHO/SEAR, 2011; WHO/WPR, 2011).

Population growth and urbanization are risk factors that can increase the burden of dengue. Population levels of more developed nations, both in urban and rural areas have and will continue to remain relatively constant; whereas less developed rural population levels are predicted to begin falling within the next decade. Urban areas within developing nations are expected to have a continued significant increase during the coming decades (Figure 23) (UN, 2009a). Population growth of these less developed nations in urban settings is primarily a result of natural population growth, although migration within the country from rural settings and immigration from other countries contributes to urbanization (UN, 2010).

The effect of urbanization has an entirely different set of outcomes depending on whether it occurs in a more or less developed nation. Developed nations have relatively strong economy which permits the government ample resources to invest in water, drainage, and sanitation infrastructure. These investments have allowed for a transition from primarily infectious diseases mortality within the state to chronic disease. Many less developed nations do not possess the resources for infrastructure expansion to keep pace within the rapidly expanding cities. The urban poor are disproportionately affected by infectious diseases as a result of disparities in healthcare, increased contact risk, and increased mobility within urban environments (Alirol, Getaz, Stoll, Chappuis, & Loutan, 2010).

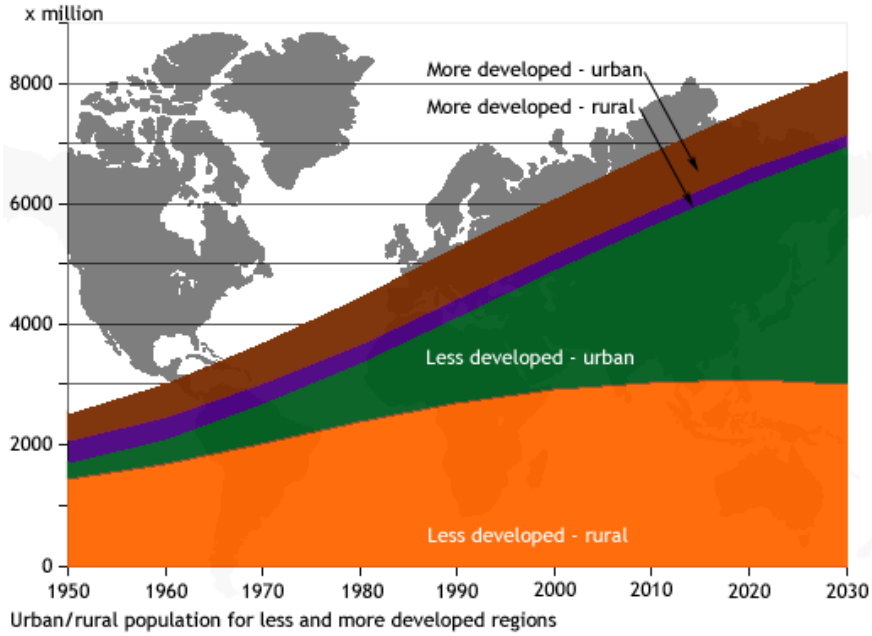


Figure 23. Historic and predicted global population growth by development and rural/urban status (UN, 2009a).

Prevention

Without a licensed dengue vaccine, control and prevention of dengue has focused on vector eradication programs. Vector control programs that have seen limited success focus on sustained local efforts to eliminate breeding sites and not large scale infrequent responses, such as communitywide fumigation (Ballenger-Browning & Elder, 2009). Modern societies produce large quantities of disposable non-biodegradable containers which accumulate and retain rainwater. Urban residences also have many containers which retain stagnant water ideal for breeding including potted plants, gutters, water storage, and air

conditioners (Kyle, & Harris, 2008). Health department education programs and public service announcements continue to attempt to educate and organize the public to eradicate dengue breeding sites; however, until a widespread behavioral change occurs in the majority of a population, dengue will continue to a public health threat.

Host

Pathophysiology

Dengue virus infection can cause a range of manifestations from asymptomatic infection to life threatening shock, hemorrhage, and organ failure (WHO/TDR, 2009). Of the100 million annually estimated dengue infections, 10-50% will develop a symptomatic response. Only a small percentage of the individuals who develop symptomatic dengue infections will be captured by surveillance systems and/or develop severe complications (Figure 24) (Kyle, & Harris, 2008). There is currently no way to determine how an individual will respond after being infected with dengue virus (Kyle, & Harris, 2008).

Following infection from a dengue infected vector, an individual will experience a four to ten day incubation period (WHO/TDR, 2009). Viremia levels reach detectable and transmittable levels approximately one day before manifestation of overt symptoms.

Symptomatic dengue disease progression is broken up into three phases: febrile, defervescence, and convalescence (Figure 25) (CDC, 2010). The febrile phase typically lasts two to seven days and is characterized by the presence of a high grade fever (WHO/TDR, 2009). Other symptomatic manifestations commonly include rash, headache and/or retro-orbital pain, nausea, vomiting, dehydration, and muscle and/or joint pain (WHO/TDR, 2009). A 'saddleback' fever pattern is not uncommon in dengue patients and should be recognized by clinicians for proper diagnosis and treatment (Rigau-Pérez *et al.,* 1998). In most individuals a significant decline in platelet count and concurrent increase in hematocrit can be observed late in the febrile phase (Figure 26) (WHO/TDR, 2009).

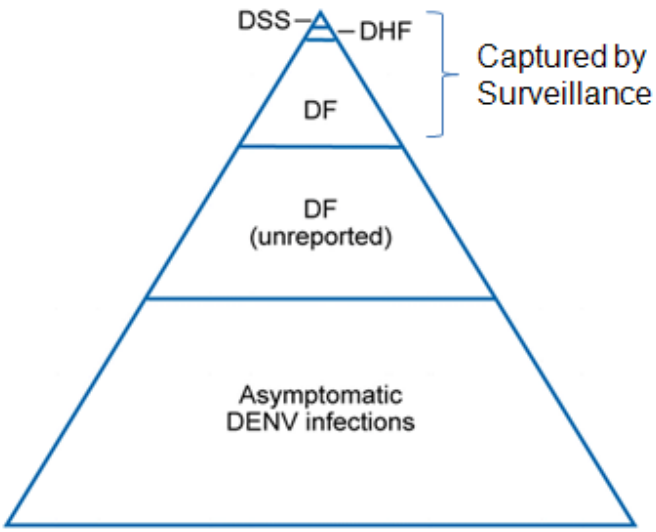


Figure 24. Breakdown of estimated 100 million annual dengue infections worldwide (Kyle, & Harris, 2008) edited.

Defervescence, also known as the critical phase, is a 24-48 hour period during which adverse clinical complications may present (CDC, 2010). The critical phase is identified with a drop in fever that may progress into below normal body temperatures in some individuals. Severe symptoms may appear during this time period include abdominal pain, fluid accumulation, mucosal bleed, lethargy or restlessness, and hepatomegaly. If left untreated, individuals may develop severe complications including vascular leakage, severe hemorrhage, and organ impairment, which may lead shock and possibly death (WHO/TDR, 2009). Adults and children have been observed to manifest differently during this time period with vascular leakage predominantly observed in children and severe hemorrhage observed more often in adults (Whitehorn & Farrar, 2010; Orthman, 2007).

The last phase of dengue illness is convalescence or re-absorption (CDC, 2010). While the convalescence phase lasts 2-4 days by WHO standards, Lum and colleagues (2008) have shown that quality of life among dengue patients does not return to approximately normal levels until almost two weeks after the beginning of illness. The finding suggests the recovery period for dengue is much longer than currently proposed (Lum, Suaya, Tan, Sah, & Shepard, 2008). During the convalescence phase, complications will begin to resolve.

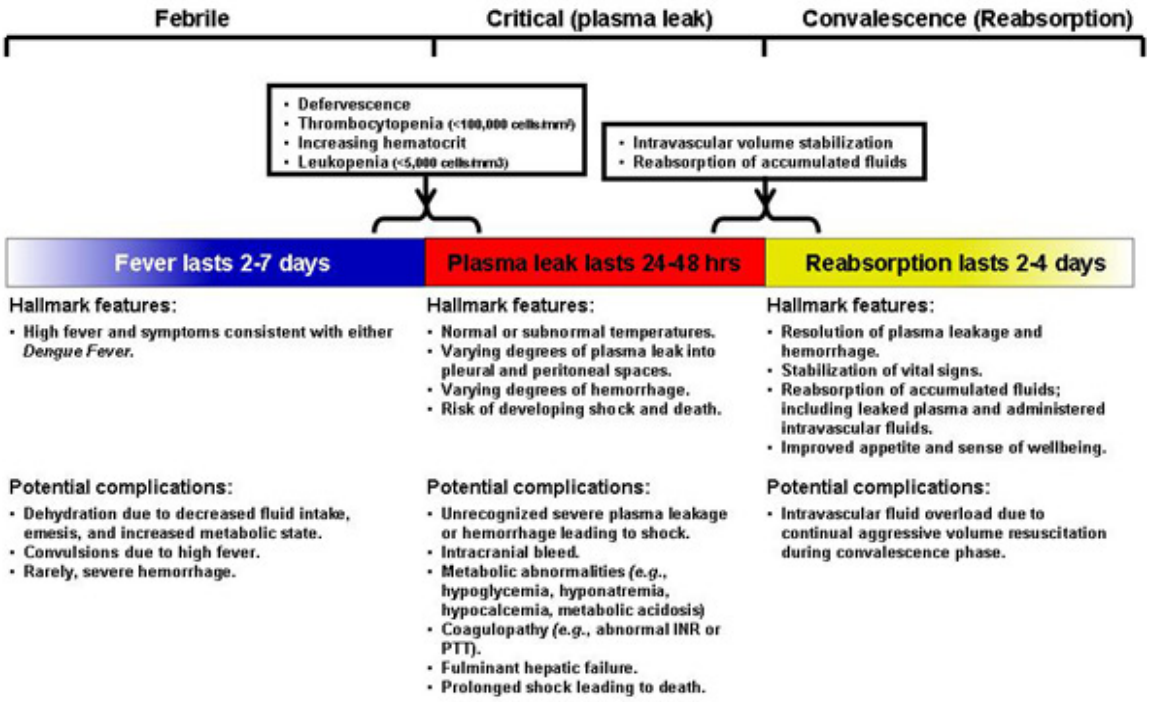


Figure 25. Phases of dengue infection (CDC, 2010).

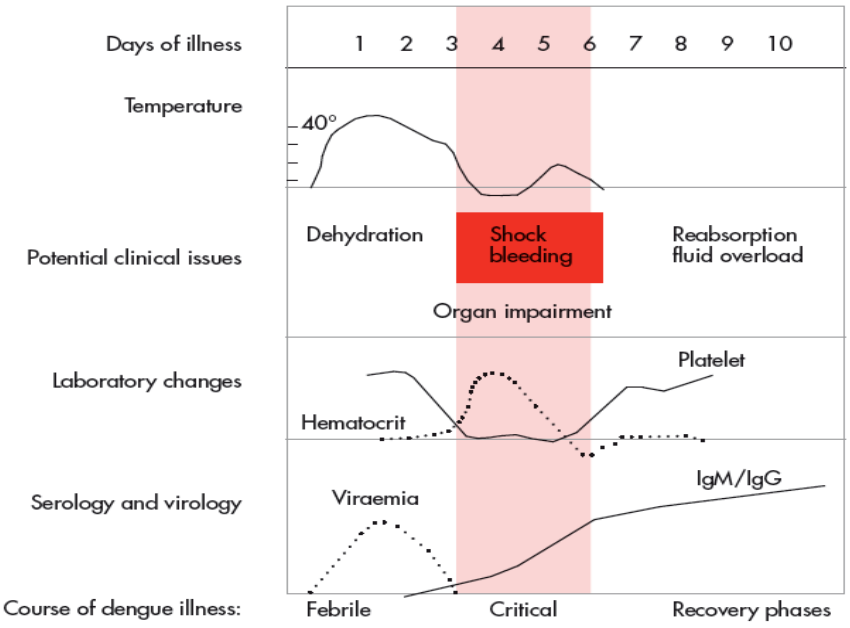


Figure 26. "The course of dengue illness" (WHO/TDR, 2009).

Intrinsic Risk Factors

Intrinsic risk factors for dengue are inherent within a person that predisposes them to biologically react in a particular manner towards a dengue infection, such as to develop symptomatic dengue, develop mild manifestations, or develop life threatening complications. Intrinsic risk factors identified through epidemiological studies to be significant for development of dengue complications include age, gender, race, host genetics, co-morbidities, and a previous dengue infection (Appanna *et al.,* 2010; WHO/TDR, 2009; Kyle & Harris, 2008; Liu *et al.,* 2008; Ooi, Goh, & Gubler, 2006; Sierra *et al.,* 2006). A secondary heterotypic dengue infection is the most significant intrinsic risk factor associated with development of severe dengue complications. Fried and colleagues (2010), found a 410% increase in odds for development of DHF in those individuals with a previous dengue infection compared to those with DF.

Diagnostic Criterion

Following outbreaks of dengue hemorrhagic fever in the Philippines and Thailand during the 1950s, WHO developed criterion for the clinical diagnosis of dengue (Rigau-Perez, 2006). Since its implementation, the dengue diagnostic criteria have undergone several revisions with the final version released in 1997 (WHO, 1997). The WHO 1997 dengue diagnostic criteria dichotomously classify dengue patients into individuals with dengue fever or dengue hemorrhagic fever

(Figure 27) (WHO, 1997). Dengue hemorrhagic fever is subdivided into four classes of which classes 3 & 4 are collectively given the term dengue shock syndrome (WHO, 1997).

After the release of the final revision of the 1997 WHO dengue diagnostic criteria, researchers and clinicians increasingly expressed dissatisfaction with the dengue diagnostic criteria (Rigau-Perez, 2006). One of the major complaints was that individuals were observed to experience severe dengue complications, but due to the overly stringent dengue classification criteria a DHF classification was not permitted (Rigau-Perez, 2006).

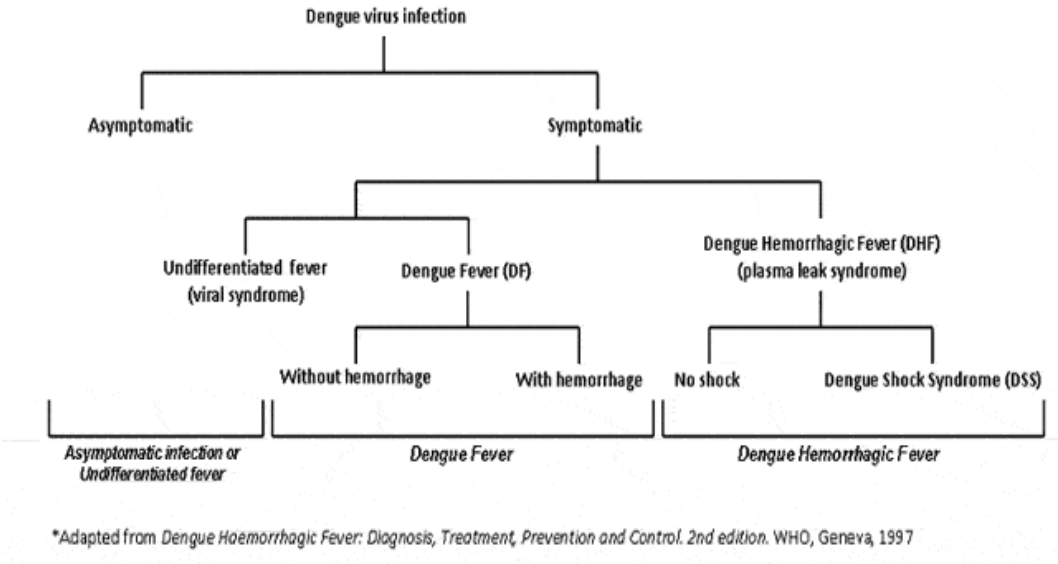


Figure 27. "Manifestations of Dengue Virus Infection" (WHO, 1997).

In 2009, the WHO and Special Programme for Research and Training in Tropical Diseases (TDR) suggested a new set of dengue classification criteria to address criticism of the 1997 WHO dengue criteria (Figure 28) (WHO/TDR, 2009). The suggested 2009 WHO dengue diagnostic criteria has been recently accepted as the new standard. Also, the new diagnostic criteria has been verified to be much more flexible in the classification of patients over the 1997 WHO diagnostic criteria (Barniol *et al.,* 2011).

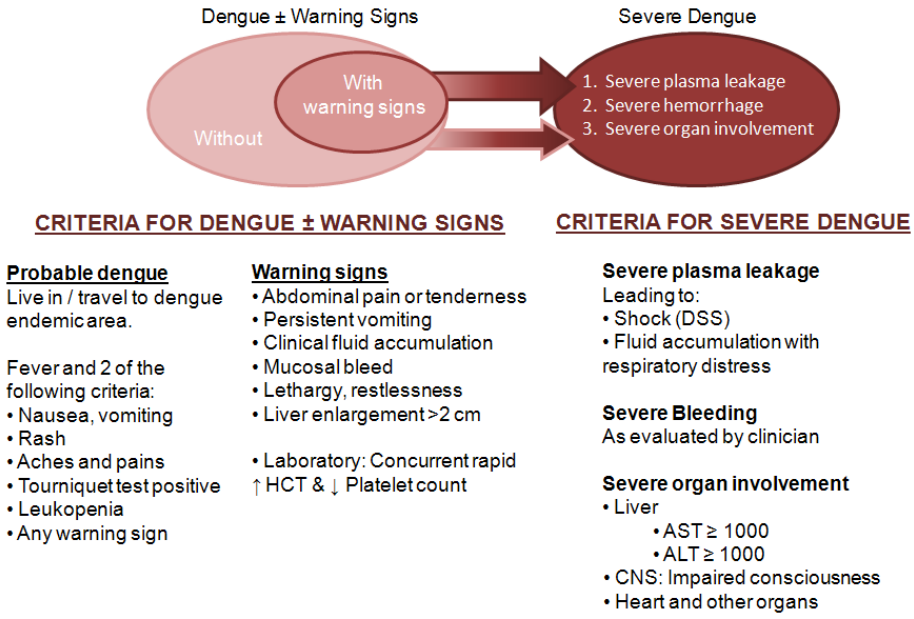


Figure 28. "Suggested dengue case classification and levels of severity" (WHO/TDR, 2009) recreated.

Treatment & Prognosis

Currently, there is no licensed medicine for dengue, and care for symptomatic dengue infection is palliative with closely managed rehydration therapy (WHO/TDR, 2009). Immediate clinical management of fluid rehydration therapy can counteract the effects of plasma leakage, hemorrhage, and organ impairment. Under trained clinical care, the dengue hemorrhagic case-fatality rate can drop below 1%; conversely, if left untreated, the case-fatality rate can climb to 20% (WHO/TDR, 2009; Monath, 2007). Dengue fever is rarely fatal and typically resolves within a week of symptomatic onset.

Prevention

Personal preventative measures to avoid a dengue infection to date have revolved solely around reducing contact with vectors as there is currently no licensed, market ready dengue vaccine. Beyond reducing breeding sites for dengue vectors in and around the home, an individual can reduce their risk infection by wearing protective clothing and/or insect repellant. These protective measures should be closely observed during dawn and dusk when dengue vectors are most active.

Corresponding in the May 2011 issue of The Lancet, Guy and colleagues update the ongoing progress of the efforts of several groups to develop a dengue vaccine. The authors report there are many different techniques being employed

to develop a dengue vaccine, including cell passage, recombinant DNA technology, and subunit implementation. In October, 2010, the first dengue vaccine went into phase 3 clinical trials. This vaccine was developed by Sanofi Pasteur and is a tetravalent vaccine that has been safely given to 5000 adults and children. The only disadvantage of this vaccine so far is that two to three injections are needed to confer protection (Guy, Almond, & Lang,2011).

Laboratory Assays

Dengue Diagnostics

Dengue infections in the acute phase mimic a flu-like illness making symptomatic clinical differentiation from other infectious diseases found in the same geographic regions as dengue extremely difficult (i.e. influenza, leptospirosis, chikungunya) (WHO/TDR, 2009). Dengue diagnostic assays have been developed to detect several analytes; however, no single assay can yet detect a dengue infection throughout the disease course. Until a single, affordable dengue diagnostic assay is developed to detect a current dengue infection, regardless of disease stage, extensive knowledge of dengue diagnostics advantages and limitations are needed to correctly use and interpret assay results. Comprehensive descriptions of technology and methodology for the detection of viremia, non-structural protein one (NS1), immune response of Immunoglobulin M (IgM) and Immunoglobulin G (IgG) have been described

elsewhere (Peeling *et al.,* 2010; Vaughn *et al.,* 2000; Innis, 1997; Vornham & Kuno, 1997). The following section will provide an overview of the presence of dengue analytes during dengue disease used in diagnostic assays.

Viremia will be detectable from approximately one day before overt disease symptoms until the beginning of defervescence, or roughly the fifth day of symptomatic dengue illness (Figure 29) (PanBioDengue, 2011). The first serological marker of a dengue infection is non-structural protein one (NS1). NS1 is present in detectable levels from Day 1 up to Day 9 of illness in both primary and secondary dengue infections.

The human immune system has a heterotypic response with respect to anti-dengue immunoglobulin M (IgM) and Immunoglobulin G (IgG), dependent on primary or secondary dengue infection status. In a primary dengue infection, IgM can be detected as early as Day 3 of illness, but usually is not detected until Day 5. Immunoglobulin M levels will peak around two weeks from the beginning of illness and will rapidly decrease to undetectable levels by three months post-infection. Immunoglobulin G will not be detected until early convalescence in a primary dengue infection, but can remain detectable for decades.

Unlike a primary dengue infection immune response, IgG will typically precede IgM detection in a secondary heterotypic dengue infection. Anti-dengue IgG may be detectable by Day 3 of illness, but is generally not until Day 5 or 6. The IgG antibody response in secondary infection is much greater than the

primary or past infection and will remain elevated for approximately one month before returning to levels observed in a primary infection.

The immune response of anti-dengue IgM is varied among individuals with a secondary dengue infection. Generally the IgM immune response is not detectable until late in the febrile phase of dengue illness. The detected response is less than observed in a primary infection and, in a minority of individuals, no response may be detected. A delayed immune response of anti-dengue IgM antibodies, during a secondary dengue infection, might occur with detectable quantities not presenting until 20 days after onset of illness.

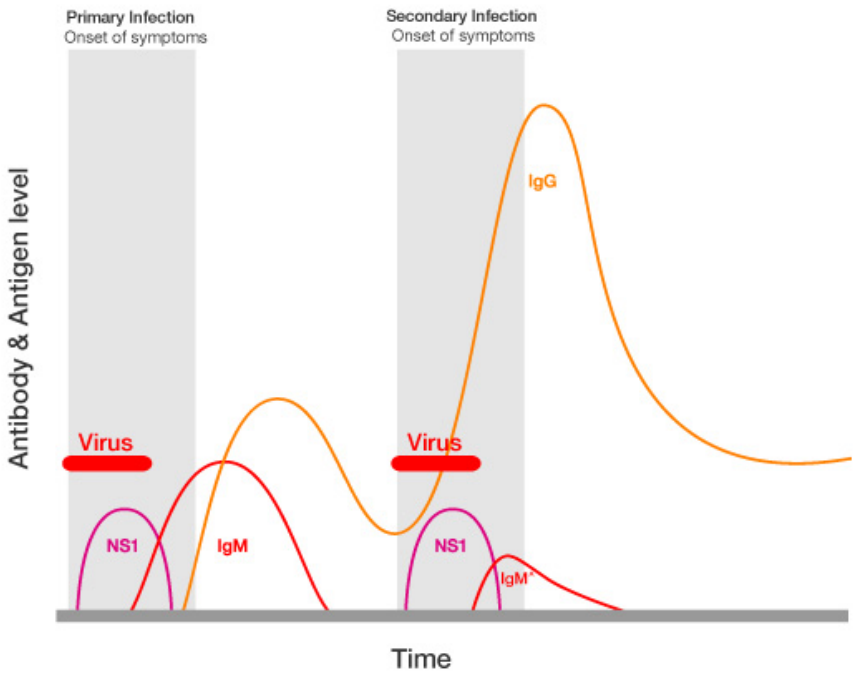


Figure 29. "Dengue infection: Immune response" (PanBioDengue, 2011).

The following sections will introduce the basic mechanics, advantages and limitations, and briefly discuss result interpretations of each of the commonly used dengue diagnostic assays. A comprehensive discussion of all dengue diagnostic assays can be found elsewhere (Peeling *et al.,* 2010; WHO/TDR, 2009; WHO/TDR, 2004).

Viral RNA Detection

One of the most recent advances in dengue diagnostics has been the development of the reverse transcriptase – polymerase chain reaction (RT-PCR) assay able to detect the presence of dengue viral RNA in patient samples in real time (Peeling *et al.,* 2010). Before one can use this new, powerful technology, it is necessary to extract the viral RNA from the patient sample. Commercialized kits can be obtained which allow for rapid simultaneous extraction from many samples. After extraction, samples can be stored for later use or immediately run in batch groups on the RT-PCR machine, with results being obtained in a few hours.

Results of the RT-PCR assay are reported as positive for dengue or indeterminate. A positive result confirms a current dengue infection and will be reported with the infecting serotype (DENV1-4). An indeterminate result may be the result of a true non-dengue sample, sample taken too late in dengue disease, improper handling or processing of patient sample, or improper execution of viral

RNA extraction and/or RT-PCR method. If results are indeterminate, follow-up samples should be requested for antigen and/or serology testing to determine if a person is truly dengue-negative.

Use of RT-PCR in dengue diagnostics allows for timely results that are highly specific and sensitive. The principal advantage of RT-PCR over other dengue diagnostic assays is the ability to confirm a dengue diagnosis with a single patient sample.

Due to the advanced technology and methods used in RT-PCR, there is need for a substantial initial investment of financial resources to acquire instrumentation and train personnel. As RT-PCR is only useful during the febrile phase, the steep financial investment may not be cost-effective for some institutions as not all patients will arrive at the hospital during the febrile phase.

Non-Structural Protein One Detection

Detection of the dengue NS1 protein is accomplished through a sandwich enzyme-linked immunosorbent assay (ELISA) (PanBioDengue, 2011). Several economical commercial assays have been developed and validated using patient serum samples, however, the use of plasma samples has yet to be established. As the NS1 protein can be detected from Day 1 up till Day 9 of illness, the NS1 ELISA provides a bridge to detect a dengue infection throughout disease course

as there is a potential gap between reduction of viremia and increase in dengue immunoglobulin (Dussart *et al.,* 2006).

Results of the NS1 kit are reported as positive, fail to detect, or equivocal (PanBioDengue, 2011). Interpretation of a positive result is that the dengue NS1 protein has been detected in the patient's serum, which is indicative of a current dengue infection. However, further serology testing on a follow-up serum sample should be completed to confirm a dengue diagnosis as there are limitations to the assay. A fail to detect result means that NS1 to dengue has not been detected; however, this does not rule out a dengue infection. Samples with results of fail to detect and equivocal should undergo a dengue IgM ELISA to further exclude a dengue diagnosis.

The NS1 ELISA is rapid and useful for detecting a current dengue infection when RT-PCR and IgM ELISA analytes may not be present in detectable quantities. The NS1 ELISA cannot by itself confirm a dengue diagnosis but can suggest the possibility of a current dengue infection. Limiting the diagnostic capability is serological cross-reactivity between dengue and other flaviviruses (Peeling *et al.,* 2010).

Immunoglobulin M Detection

Detection of dengue IgM antibodies is accomplished through a capture ELISA. Like the NS1 ELISA, the IgM ELISA has been developed using patient

serum samples. As anti-dengue IgM antibodies are present in detectable levels from approximately Day 5 of illness up to three months post-infection, they provide an ideal means of identifying individuals with a recent dengue infection (Peeling *et al.,* 2010).

Results of the IgM ELISA are reported as positive, fail to detect, and equivocal. A positive result indicates that the IgM to dengue has been detected. Equivocal results should be re-run with a follow-up sample. The interpretation of a fail to detect result is dependent on the onset date of clinical symptoms. If the sample was collected 20 days after dengue-like disease onset, then the patient most likely did not have non-dengue. However, if the sample was collected less than 20 days from onset of dengue-like clinical symptoms, then the result is reported as IgM to dengue not detected, and a request is made for a follow-up sample as the patient may have not yet seroconverted.

The dengue IgM ELISA is affordable, easy to run, and does not require large investments of financial resources to implement. However, due to the inherent duration of the anti-dengue IgM antibodies in the body, dengue cannot be confirmed with a single positive IgM ELISA. To confirm a dengue diagnosis using IgM ELISA, two temporally spaced patient samples must be obtained. As anti-dengue IgM titers do not reach their peak until approximately two weeks after onset of symptoms, an increasing trend or seroconversion will confirm a dengue diagnosis in temporally-spaced paired-samples.

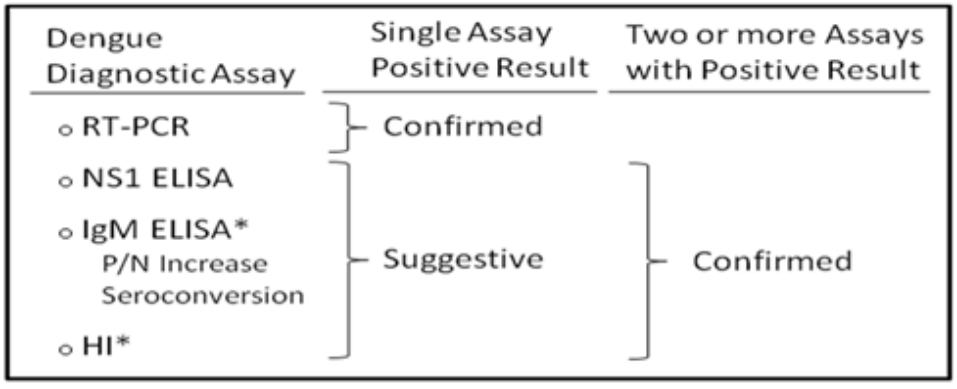
Immunoglobulin Detection

The dengue haemagglutination inhibition (HI) quantitates the patient serum anti-dengue immunoglobulin (Ig) irrespective of Ig isotype (i.e. IgA, IgM, IgG) (De Paula & Fonseca, 2004). Recently, with the development of sensitive IgG and IgM ELISA, HI popularity in dengue diagnostics has decreased as it is a time and labor intensive process (minimum two days). HI also requires paired patient sera with removal of non-specific inhibitors and agglutinations from sera. However, minimal equipment to complete the assay, reliability, and that the assay is well-standardized keeps the dengue HI assay as the gold standard in determining if a dengue infection is primary or secondary in nature. The distinction between primary and secondary dengue infection is possible as sustained levels of serum anti-dengue Ig are significantly different in primary and secondary cases.

Findings will be reported as positive for dengue with a primary or secondary dengue infection or dengue not detected. Patient samples with an acute sample HI titer <1280 and a convalescence sample HI titer <2560 are considered primary infections. Secondary infections will have an acute sample titer of <2560 and a convalescence sample titer of >2560 (De Paula & Fonseca, 2004).

Laboratory Dengue Confirmation

Currently there is no laboratory assay capable of detecting a dengue infection regardless of disease progression. Without this universal dengue tool, there is a need to fully understand dengue diagnostics to aid in diagnosis and correctly report cases for surveillance. A single sample positive RT-PCR result can confirm a dengue diagnosis (Figure 31) (Peeling *et al.,* 2010). If the RT-PCR result comes back as indeterminate, then antigen and serology tests must be completed to confirm a dengue diagnosis. Potential cross-reactivity of antigens and long lifespan of serological components make necessary the need to obtain two positive results to confirm a dengue diagnosis. The use of one positive sample of antigen and serological dengue assays can suggest a dengue infection, but it is an incorrect practice to laboratory confirm a dengue infection.



*Requires paired samples.

Figure 30. Criteria to laboratory confirm a dengue infection (Peeling *et al.,* 2010).

METHODOLOGY

Study Location

The study location was Federal Hospital Ampang in Ampang, Selangor, Malaysia.

Country

Malaysia, located in Southeast Asia, is comprised of an area slightly larger than the US state of New Mexico at 329,847 sq km, is split into the two distinct land masses of Peninsular Malaysia and Borneo Malaysia by the South China Sea (CIA, 2011). The majority of Malaysia experiences a tropical climate; however, sub-tropical conditions exist in the interior mountains in both Peninsular and Borneo Malaysia. Annual monsoons drive changes in climatic conditions, typically seen in the southwest from April to October, and the northeast from October to February (CIA, 2011). Total annual rainfall can accumulate to 2,427millimeters (95.6 inches) in Peninsular Malaysia, though rainfall totals can be double this amount in Borneo Malaysia (WWIS, 2011). Annual average temperatures have low variability throughout Malaysia; Kuala Lumpur annual high temperatures range from 31.5ºC to 33.2ºC (88.7F to 91.8F), and low temperatures range from 22.5ºC to 23.9 ºC (72.5F to 75.0F) (WWIS, 2011).

The 2010 Malaysian Census reported the Malaysian population to be 28.3 million with a 1.04 male/female ratio. The main racial backgrounds of the Malaysian population include: Malays (60.3%), Chinese (22.8%), Indians (6.8%), and Others (10.0%, including expatriates). The Malaysian population age groupings are skewed left with 27.2% aged 0-14, 68.1% aged 15-64, and 4.7% aged 65 and over in 2010 (Dr. J. Sathar, personal communication, March 2011).

The average Malaysian household annual income is an estimated Malaysian Ringget $44,500 (~US$14,700) with 5.1% (est. 2002) of the population below the poverty line (CIA, 2011). Literacy rate for the Malaysian population is 88.7%, with females staying an average of 13 years, one year longer than males, in formal education (CIA, 2011).

Malaysia is divided into 13 federal territories, of which the nine western federal territories are the most populated (Figure 31) (Encyclopedia Britannica. 2009).

City

The formal capital city, Kuala Lumpur, resides in Kuala Lumpur Federal Territory in southwestern Peninsular Malaysia. Surrounding Kuala Lumpur Federal Territory is the most populous federal territory, Selangor, with 5.1 million individuals in 2010 (Dr. J. Sathar, personal communication, March 2011). Selangor, save the new federal territory and governmental capital of Putra Jaya

(growth rate: 17.8%), is the fastest growing federal territory with a growth rate of 3.17% in 2010 (national growth rate: 2.17%) (The World Bank, 2011). The District of Ulu Langat is the westernmost of the 9 districts which comprise Selangor.

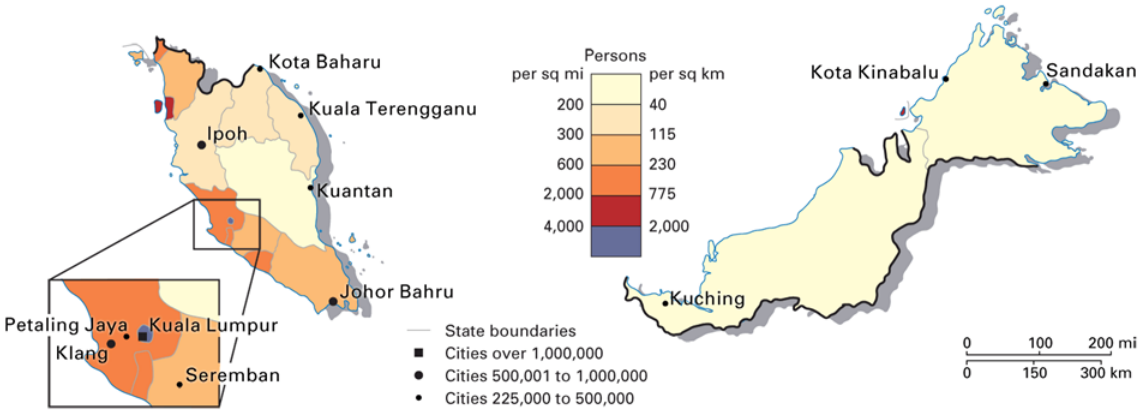


Figure 31. Population density of Malaysia in 2009 (Encyclopedia Britannica. 2009).

Hospital

Federal Hospital Ampang is located in the subdivision of Ampang, one of seven subdivisions which makeup the District of Ulu Langat. Statistics from the 2010 Malaysian Census for the District of Ampang will not be available till 2012 (Dr. J. Sathar, personal communication, March 2011). In 2000, the District of Ampang population numbered 357,925 which broken down by racial background:

Malay 45.2%, Chinese 40.2%, Indian 8.5%, Other 6.1% (Dr. J. Sathar, personal communication from Ministry of Statistics, March 2011).

The Malaysian healthcare system is similar to the United States two-tier system of public and private services (MMOH, 2011). Easy access to healthcare is provided to anyone in a public hospital after a co-pay of Malaysian Ringget $1 (~US$0.33 March 2011). Selangor currently has 20 private hospitals and 12 public hospitals (MMOH, 2011); Federal Hospital Ampang is a public hospital with 560 beds (K. Khalid, personal communication from Hospital Ampang, March 2011). Opening in 2006, Hospital Ampang is modern 8-story structure complete with a two-story out-patient clinic, tri-stage (general, semi-critical, and critical) emergency department, comprehensive and rapid clinical laboratory with modern equipment, blood bank, and transplant capabilities. Hospital Ampang retains a two-tiered patient room structure with dormitory style multi-bed wards throughout the hospital and private rooms located on the 8th floor.

Dengue Ward

Dengue has become a major burden to the healthcare system in Malaysia. At Hospital Ampang and other hospitals in Malaysia (e.g. Klang Hospital), to facilitate a standard of care for individuals suspected or confirmed to be infected with dengue, a dengue ward has been established. Admitted patients from the emergency department, clinics, other departments in Hospital Ampang and

surrounding area hospital transfers who are suspected or have been confirmed to have dengue, are transferred to the dengue ward as standard hospital procedure. Patients who are in critical condition (i.e. hypovolemic shock), will be transferred to the Intensive Care Unit (ICU); however, after dengue cases have been stabilized, they are transferred back to the dengue ward. The dengue ward is a dormitory style ward with 28 beds (2 isolation rooms) separated by gender. Under epidemic conditions, additional beds can be included as beds are generously spaced. In-ward office and kitchen space allow for continued presence of medical staff.

Due to the cyclical pattern of dengue outbreaks, dengue patient loads will vary potentially leaving the majority of the dengue ward unused. To ensure the hospital efficiently uses all resources, patients without dengue will be transferred to the dengue ward for care. Patients transferred typically have infectious diseases (tuberculosis, HIV, leptospirosis, etc.); although chronic diseases (heart disease, diabetes, etc.) can also be transferred. To rapidly identify dengue cases wall name tags above the patient beds are green for dengue and blue for non-dengue.

Treatment & Care

Treatment and care of dengue ward patients are performed by hospital administration, senior doctors, junior doctors, head nurses (‘Sisters’), nurses,

junior nurses, medical technicians, student nurses, and janitorial staff. Daily morning medical 'rounds' are completed by the single senior doctor overseeing the ward, two to three junior doctors (known as 'housemen'), head nurses, and nurses. Housemen are rotated between medical wards on a monthly schedule for comprehensive medical training. Housemen and nurses are responsible for recording all medical information in the hospital electronic database. Three carts with laptops and wireless internet allow for bedside note taking, while three additional desktops provide comfort for more lengthy entries. Hardcopy medical charts including vital statistics are kept updated at each patient's bedside by junior nurses and student nurses in case of power or database failure. Hardcopy charts are stored upon patient discharge.

Junior doctors and/or nurses draw blood for clinical assessment four times a day at the six o'clock and twelve o'clock hours or as directed by the senior doctor. Clinical laboratory tests typically include blood, kidney and liver profiles. A blood profile measures venous levels of the following: hemoglobin, hematocrit, platelet count, total white cells, neutrophils, lymphocytes, monocytes, eosinophils and basophils. A liver profile assesses venous levels of total bilirubin, alkaline phosphatase, total protein, albumin, globulin and alanine aminotransferase. A kidney profile indicates venous levels of urea, sodium, potassium chloride and creatinine.

All patients are administered intravenous hydration saline solution, which are monitored to ensure patients are properly hydrated throughout disease

course. Blood transfusions and platelet transfusions are no longer considered for dengue treatment in the dengue ward (practice changed in early 2010); different practices may be present in the ICU. The tourniquet test is not administered as standard dengue diagnostic procedure. Patients are not discharged before the end of the 48 hour defervescence phase of disease and may remain admitted for longer periods of time (i.e. days or weeks) if complications arise (e.g. severe viral hepatitis).

Study Population

The study is a subset within a larger cross-sectional medical microbiology study entitled: *Genetic and Immunological Profiles during the Progression of Dengue Disease* (further referred to as 'source study'). The source study is under the direction of Professor Dr. Shamala Devi of the Medical Microbiology Department, Faculty of Medicine, at the University of Malaya in Kuala Lumpur, Malaysia. The source study was conducted from June to December 2010.

To fully understand this study and the study population, it is necessary to first describe the purpose and study design used in the source study. The source study was envisioned to fill a gap in the current dengue literature of a comprehensive clinical and laboratory profile of dengue disease to aid in the discovery and development of diagnostic assays, treatment, and vaccine.

The source study targeted the fraction of the dengue infected population who manifest with complications requiring medical intervention, as they are most in need of medical breakthroughs.

The source study employed a cross-sectional design; however, the design does not fit the classical form of the study type. Infectious diseases are unique from chronic diseases in that if a patient develops, say dengue, it is known they have been exposed to the dengue virus when they arrive at a hospital for treatment. Consequently, the source study population consists of all new dengue cases, beyond patients who were suspected to have dengue on study recruitment and were later diagnosed with a similar manifesting disease (e.g. leptospirosis). Exposure is therefore not regarded in the classical sense as exposure to the infecting agent or not, but is perhaps a pre-existing condition, such as previous infection with dengue virus that can be determined through laboratory analysis. Outcome is also dissimilar to the classical case and control. The outcome might be defined, for example, as monocyte count or presence and absence of hemorrhage. The discharge diagnosis was used in the source study.

The source study design also deviates from the classical form of a cross-sectional design by the inclusion of a longitudinal dynamic in data collection. As the purpose of the primary study was to develop a profile of dengue disease progression, patients were followed throughout their hospitalization. For the source study, patient clinical and laboratory data was collected at three time points with one point in each of the three phases of dengue disease (febrile,

defervescence, and convalescence). Even with the longitudinal dynamic of the source study, the design remains a cross-sectional study due the exposure and outcome of interest being determined at the same time.

The present study only assesses patient data within the defervescence phase of illness. Demographics of the source study are presented in the RESULTS section.

Study Design

The study design is an analytic cross-sectional study that has been subset within the source study completed from June to December 2010. Subsetting the study within the source study allows for efficient use of resources as preliminary empirical justification for a standalone study has not yet been demonstrated. Few epidemiological studies have investigated the relationship of monocytes during dengue disease. No studies were found in the literature review that examined the association of monocytes with primary and secondary dengue infection status.

The target population of the study includes all individuals aged 15 years or older, who are susceptible to dengue and would develop severe complications as a result of infection, and live in Malaysia. The target population is the population to which the study findings would ideally be inferred. The source population, the population from which individuals will be selected for study inclusion, includes

individuals aged 15 years or older that have acquired a dengue infection and came to Hospital Ampang for medical treatment from June to December 2010. The study eligible population is comprised of those individuals from the source population who meet the study inclusion and exclusion criteria.

Study inclusion criteria included admission to Hospital Ampang for dengue, and age of 15-17 years with written parental permission, or age of 18 and above. The study exclusion criteria excluded from the study those potential study participants that did not have the ability or willingness to provide informed consent in English, Malay, Chinese or Tamil; did not have consenting permission of the overseeing clinician for patient inclusion; or did not have a recorded venous absolute monocyte count in the Hospital Ampang computerized patient database for the defervescence stage of dengue disease.

The study inclusion/exclusion criteria have allowed for targeting of a study population that will allow for ideal analysis of the association of interest. Defervescence is the critical time period during which severe dengue complications manifest. Restricting the study to only hospitalized patients during defervescence, permits assessment of the relationship of interest on a highly burdened population that may provide insights that may not be observable in other populations or dengue disease phases.

Dengue literature has primarily focused on individuals less than 15 years of age due to the suspected fragility of their undeveloped capillaries resulting in

increased risk for development of severe dengue manifestations (Kyle & Harris, 2008). The UN defines “children” as those less than 18 years of age (UN, 1990); however, for statistical purposes the UN has defined “youth” as those between the ages of 15-24 years (UNESCO, 1985). A study population consisting of youth and adult, non-youth was recruited for the study as severe dengue has increasingly effected these populations.

Unlike other infectious diseases (i.e. human immunodeficiency virus or tuberculosis), dengue is a non-chronic disease making the study population composed of all incidence cases. In this study, the study exposure is not defined as having been exposed to the disease agent, but having a current primary or secondary dengue infection. The use of the hemagglutinin inhibition laboratory assay allows for determination of a patient’s dengue infection status (primary or secondary).

Studies have observed that a previous dengue infection is a risk factor for development of complications during dengue disease. Biological pathway hypotheses have suggested that the primary dengue infection alters the immune system response to a secondary heterotypic dengue infection. Using dengue infection status as the study exposure will permit observation of the primary objective on whether or not the predisposition has an association with venous absolute monocyte count.

Unlike the classical epidemiologic cross-sectional study outcome of case and control, the study outcome is a difference in the venous absolute monocyte count. The study outcome has not been dichotomized by a cut point because no previous estimate of the relationship under investigation was found in the literature review. The study's null hypothesis is that there is no difference in venous absolute monocyte count by dengue infection status. Absolute monocyte count is a reported clinical laboratory result given as part of a normal blood profile. As a blood profile is a standard set of clinical laboratory assays administered to dengue patients in Hospital Ampang, most dengue patients were expected to undergo a blood profile during defervescence. Monocytes are of interest due to their role in both innate and adaptive immunity.

The literature review identified several risk factors which needed to be included in the study analysis due to their association with the study exposure and outcome, not being part of the hypothesized causal pathway, and potential for being present in different degrees between study groups. These risk factors are race, age, and gender. Race was categorized by the major race observed in the Malaysian population, which are Malay, Chinese, Indian, and Other. Gender was categorized as male or female. A review of the dengue literature found many different cuts points by which age was categorized (groupings of 2, 3, 5, & 10 years) with no clear indication given for the reasoning behind the selection. Whereas studies investigating children with dengue used smaller interval cut points, studies investigating adults tend to categorize the age demographic by

groupings of 5 or 10 years. The study assessed the age demographic by both 5 and 10 year groupings; however, only the 10 year groupings are displayed and assessed in the regression analysis as no significant results were observed using the 5 year grouping. Age was categorized as 15-24 years, 24-34 years, 35-44 years, and 45 years and above. The last grouping was set as 45 years of age or above because few individuals were recruited for the study above 55 years of age. Age was also assessed as a continuous variable to determine if the cut points were creating or masking an association.

Known risk factors which were unable to be assessed in the analysis included infecting viral serotype, socioeconomic status, and education. No data was available on these risk factors as current practice at Hospital Ampang does not take this information from the patient and the hospital laboratory does not possess the necessary assay.

To consider the influence of these unattained risk factors a surrogate variable was used. As Hospital Ampang is a new hospital (opened in 2006) and is a transfer hospital for other major diseases (i.e. thalassemia), there is potential that patients will travel themselves or be transferred to Hospital Ampang if they have severe dengue complications. Distance between patient residence and Hospital Ampang, dichotomized at 12.5 kilometers was assessed for being a risk factor. The 12.5 kilometer radial boundary was arrived at by averaging the distance between Hospital Ampang and the closest public hospitals. This cut-off value will allow for approximate dichotomization of those individuals who are

travelling to the nearest hospital and those who are travelling extra distance to receive care at Hospital Ampang. The use of this surrogate variable was not seen in the literature review and careful consideration is needed whether or not to include in the final regression models as results maybe artificial.

The study secondary objective was to assess the relationship of absence or presence of hemorrhage/vascular leakage with venous absolute monocyte count. The literature review found few studies in which monocytes (absolute or percent) were found to be significantly associated with the outcome of the study, which is the absence or presence of hemorrhage/vascular leakage, when using the standard dengue classification criteria. A hypothesis was formed that differences in absolute monocyte count maybe associated with severe dengue complications, but is unobservable or masked most of the time when using the dengue diagnostic criteria as an outcome. Excessive effusion is the primary cause of death from dengue with vascular leakage and hemorrhage being the principal manifestations.

The secondary objective analysis uses the absence or presence of hemorrhage/vascular leakage as an outcome with the aim to determine if an association exists with absolute monocyte count. Hemorrhage was defined as presence of spontaneous gum bleed, *e*pistaxis, menorrhagia, hematemesis, subconjunctive hemorrhage, renal hemorrhage, or melena. Vascular leakage was defined as presence of pleural effusion, ascites, increase or decrease of hematocrit by 20%, hypotension (systolic blood pressure <90mmHg), or

narrowed pulse pressure (<20 mmHg) (WHO/TDR, 2009). Risk factors for the association included age, race, gender, dengue infection status (primary or secondary), and distance from patient's residence to Hospital Ampang.

Loss to follow-up in the study could have occurred as a result of the patient no longer wanting to be part of the study, and medical staff not taking study samples. To ensure patients continued to take part in the study, study recruiters took significant effort to explain to the patient the value of the study. As recruiters were in the hospital every weekday for several hours, they were encouraged to talk with participants providing them with anti-dengue educational material made and provided by the Malaysian Ministry of Health. This interaction served to provide an opportunity for the patient to ask any further questions about the study and dispel any worries about the risks of study participation the patient may have developed.

Loss to follow-up as a result of medical staff not taking samples necessitates maximum input on study staff to overcome the demanding and variant nature of medical care. The primary obstacle to overcome occurred at the beginning of every month, when junior doctors transferred assigned wards. This required study staff to inform and then train the new medical staff on the procedures of the study. To ensure minimum loss to follow-up, the study blood draws were incorporated within the established daily blood taking routine. After the study staff had recruited patients they would label the appropriate blood collection tubes and place them in order on the blood collection cart, which was

used for all hospital blood collection. The study staff then verbally communicated with the doctors on how many tubes and patients needed to be drawn for the study during the next scheduled collection time.

No financial incentive was provided to participants. To reduce the risk of potential infection and discomfort of the patient, the study blood draw was added on to the planned hospital blood draw. Taking blood samples in this manner allowed for patients to participate without having to endure any additional needle sticks than what was required by the hospital. The benefit provided by the study was to report the results of the dengue RT-PCR testing conducted at the University of Malaya laboratory. The RT-PCR testing was part of the primary study design and the patient was not charged for testing. RT-PCR results were made available to ward doctors to explain results to patients. Hospital Ampang did not have RT-PCR capabilities, but patients who did not want to participate could obtain dengue infection status through anti-dengue IgM testing routinely completed by the Hospital Ampang clinical laboratory. No additional needle-sticks and free laboratory testing allowed for the benefits of study participation to outweigh the risks to the study participation.

Data Collection

Data collection for both the source study and present study were carried out using the same protocol. The following section presents an overview of the protocol for clinical and laboratory data collection.

Clinical Data

After patients were admitted to the dengue ward in Hospital Ampang, study recruiters approached ward doctors to establish medical consent for study participation. If the doctor gave medical consent, the patient was approached for informed consent. After successful recruitment of the study participant, the hospital identification number was recorded on a standardized study participant clinical data form. The patient clinical information was obtained using the study participant hospital identification number in the hospital electronic database system. Use of the standardized clinical data form allowed for consistent recording of all study participant data. The study participant clinical data forms were transferred back to the University Malaya laboratories of Professor Dr. Shamala and input into a database using Microsoft Excel© (Redmond, WA). The electronic database was saved on a single computer and backed up using an external hard drive.

Laboratory Data

The participant study blood sample tubes were collected immediately from the dengue ward blood draw cart after medical staff had completed drawing blood. Patient blood collection tubes were then stored in an onsite 4ºC refrigerator before being transferred by cooler and gel freezer pack to the University Malaya laboratory of Professor Dr. Shamala Devi within 4 hours of the blood draw. Upon arrival the samples were immediately processed, appropriately aliquoted, and stored at -80ºC for long-term storage. Received samples were recorded in a hard copy notebook to document their presence in storage.

Samples were assessed for the presence of dengue viral RNA, dengue NS1 protein, and anti-dengue immunoglobulin M and G. Discussions and protocols for the use of dengue diagnostic assays can be found elsewhere (WHO/TDR, 2004; PanBioDengue, 2011). Results of the assays were recorded in an electronic database and back-up on an external hard disk.

Statistical Analysis

The laboratory and clinical databases were cleaned and analyzed in SAS© (Cary, NC). Frequency and univariate procedures were used to identify

missing values and outliers. All suspected errors were checked against hard copy documents.

Chi-square tests for independence and Fisher's exact test, used to examine if two populations contain the same proportion of observations of a single variable, and independent sample t-tests, used in the comparison of two populations' mean on a single variable, were used in the assessment of variables across study populations. Linear and logistic regression models were used in the assessment of the study primary and secondary objectives, while allowing for adjustment of the effects from independent risk factors on the relationship of interest.

Sample Size Calculation Statement

A sample size calculation is a necessary component of an epidemiological study and should be carried out during the planning phase of the study to ensure efficient use of limited public health resources. As this study was a subset within the source study, it was not the main consideration during study planning phase. A power calculation was conducted retrospectively to determine if sufficient power existed for the analysis (see RESULTS).

RESULTS

Retrospective Power Calculation

The retrospective power calculation was completed to ensure that there are enough study participants to detect an association above a chance finding using estimates extracted from the literature. While not ideal, the retrospective power calculation is an acceptable practice, but should be avoided if possible.

Literature on youth or adult population monocyte levels during defervescence by primary and secondary dengue infection status was not found in the literature review. In fact, monocyte levels by primary and secondary dengue infection status were not found among any population. This again illustrates the need for the study, but also the difficulty in finding an acceptable estimate to base the retrospective power calculation.

All secondary heterotypic dengue infections do not result in development of dengue hemorrhagic fever, while some primary dengue infections have severe complications. The use of the dengue clinical diagnostic classification in the study retrospective power calculation is imperfect, but remains the closest available option as secondary heterotypic dengue infection has been consistently observed to be a risk factor for severe dengue manifestations (WHO/TDR, 2009).

Green and colleagues (1999) presented findings of absolute monocyte count among 51 Thai children (<15 years of age) by clinical dengue diagnosis (dengue fever (n=29) and dengue hemorrhagic fever (n=22)) during defervescence. The dengue fever population had an average absolute monocyte count of 193 ± 119, while the dengue hemorrhagic fever population was 226 ± 196.

The power calculation employed an independent sample for means two-sided formula, as previous hypothesis were not available to justify the use of a one-sided test. A 0.05 significance level, or odds that the observed is due to chance, was used. Population estimates of 193 and 226, along with a standard deviation for the population of 152 (averaged, ((119*29) + (226*22)/51)) were used from Green and colleagues (1999) findings. With a study population of 197 participants, the estimated power is 0.87. This indicates that the odds that an association will be observed when one actually exists are 87%. The power calculation was completed using SAS® and checked in an online power and sample size calculator (Statistical Solutions, 2011).

Demography

To aid in determining the external validity of the study, the source population and source study demography will be described first followed by the study demography to allow for comparisons. The source population of the study

included all individuals above the age of 15 years who would come to and would be hospitalized at Hospital Ampang for a dengue infection from June to December 2010. Information on this exact population could not be obtained; however, demographic information was acquired on all Hospital Ampang dengue patients, in- and out-patient, during the 2010 calendar year. While not the exact source population for the primary study, the information provides a very good estimate on the entire population that would come to Hospital Ampang for treatment from a dengue infection.

The source population consisted of 16,367 cases of dengue with one death reported from Hospital Ampang during the 2010 calendar year. Deaths from dengue remain very low at Hospital Ampang with only two and three occurring in 2008 and 2009, respectively. The single 2010 dengue death at Hospital Ampang occurred before the June start of the study. Dengue cases at Hospital Ampang were 53.3% male and 62.0% Malay; 22.8% Chinese; 9.8% Indian; and 5.4% Other during 2010. The age distribution was 33.4% aged 15 to 24 years, 32.3% aged 25 to 34 years, 17.9% aged 35 to 44 years, and 16.5% aged 45 years and older (Table 1) (J. Sathar, personal communicate, April 2011).

During the data collection time period of June through December 2010, the source study approached 381 patients at Hospital Ampang who met the study inclusion/exclusion criteria, of which, 94 declined to participate. Patients who refused to participate (Source Study Refusals) tended to be male (53.3%);

Malay (62.0%); aged 15 to 24 years (35.7%); and lived within 12.5 km of Hospital Ampang (83.9%) (Table 1).

With a participation rate (of those individuals who met inclusion/exclusion criteria and were asked to participate, those who agreed) of 80.2%, there were 287 individuals who entered the source study (study entrants). Four individuals refused to participate after they entered the source study and the medical staff did not draw blood serum samples for seven samples resulting in the participants' inability to contribute to the study data. With a retention rate (of those individuals who entered the study, those who contributed to the study data) of 96.2%, the final number of study participants for the source study was 276. Source study participants were more likely to be male (63.0%); Malay (61.6%); aged 15-24 years (38.1%); and lived within 12.5 km of Hospital Ampang (83.0%) (data not shown). Source study participants were admitted to Hospital Ampang, on average, 4.08 ± 1.71 days after self-reported onset of illness and had a length of hospitalization of 4.26 ± 2.57 days. Further descriptive statistics on the source study by blood profile, liver profile, kidney profile, and vital statistics profile are presented in Appendix B.

Of the source study participants, 22 were not diagnosed with dengue. Non-dengue diagnosed individuals were more likely to be male (81.8%); Malay (68.2%); adult, aged 24-34 years (45.5%); and lived within 12.5 km of Hospital Ampang (86.4%) (data not shown). Source study participants not diagnosed with dengue were admitted to Hospital Ampang, on average, 4.19 ± 3.12 days after

self-reported onset of illness and had a length of hospitalization of 4.47 ± 1.92 days.

Of the source study participants, 248 were diagnosed with dengue and above the age of 15 years. Dengue diagnosed individuals were more likely to be male (63.0%); Malay (61.6%); aged 15-24 years (38.3%); and lived within 12.5 km of Hospital Ampang (83.0%) (Table 1). Source study participants diagnosed with dengue were admitted to Hospital Ampang, on average, 4.07 ± 1.58 days after self-reported onset of illness and had a length of hospitalization of 4.24 ± 2.61 days.

The study was a subset of the data from the source study by restricting the study population to only those participants who were laboratory confirmed with a dengue infection, and that had a recorded absolute monocyte value during the defervescence phase of illness. The study population consisted of 197 participants of which the majority were male (62.7%); Malay (64.0%); aged 25-34 years (46.3%); and lived within 12.5km of Hospital Ampang (81.2%) (Table 1). Study participants were admitted to Hospital Ampang on average, 4.07 ± 1.55 days after self-reported onset of illness, and had a length of hospitalization of 4.54 ± 2.73 days.

The chi-square test for independence was used to examine if the study population, source study participants (with a dengue diagnosis), and source study refusals contained the same proportion of observations by gender, race,

age, and distance between patient residence and Hospital Ampang. No significant differences were detected among the populations at the 0.05 level of significance.

Table 1. Demographics of Study Participants, Source Study Participants by Dengue Diagnosis, Source Study Non-Participants, and Source Population in Hospital Ampang, Selangor, Malaysia, 2010.

	Study Participants		Source Study Participants*		Source Study Refusals#		Source Population^	
	N	%	*N*	%	*N*	%	*N*	%
Total	197	77.6	248	90.0	94	100	16367	100
Gender								
Male	124	62.9	152	61.3	49	53.3	9503	58.1
Female	73	37.1	96	38.7	43	46.7	6864	41.9
Race								
Malay	126	64.0	151	60.9	57	62	9272	56.7
Chinese	44	22.3	57	23.0	21	22.8	3678	22.5
Indian	13	6.6	17	6.9	9	9.8	2081	12.7
Other	14	7.1	23	9.3	5	5.4	1336	8.2
Age (years)								
15-24	74	37.6	95	38.3	30	35.7	4495	33.4
25-34	69	35.0	85	34.3	25	29.8	4335	32.3
35-44	28	12.2	36	14.5	15	17.9	2399	17.9
>45	26	13.2	32	12.9	14	16.7	2213	16.5
Distance&								
≤ 12.5	160	81.2	203	81.9	73	83.9	-	-
> 12.5	37	18.8	45	18.2	14	16.1	-	-

* Dengue clinical diagnosis only.
# Difference to total reflect information refusal.
^ Hospital Ampang in-patient and out-patient dengue cases during 2010; Includes 2,925 children (<15 years); Total for Age excludes children.
& Distance between patient residence and Hospital Ampang in kilometers.

The source population was not included in chi-square analysis for gender and race as 17.9% of source population summary statistics obtained consisted of

children whose information could not be separated from those aged 15 years and older. Chi-square tests for independence did not detect a significant difference between the source population and all other populations in Table 1 by age.

The majority of participants within the study population had contracted a secondary dengue infection (79.2%) (Table 2). The gender ratio (male: female) was 1.56:1.0 and 1.74:1.0 for secondary and primary dengue infections, respectively. Among both primary and secondary dengue infection populations, the majority of participants were male, Malay, and lived within 12.5 km of Hospital Ampang. The majority of individuals experiencing a primary dengue infection were aged 25-34 years, whereas those experiencing a secondary dengue infection were aged 15-24 years.

Using chi-squared test for independence and fisher's exact test were appropriate, there was no significant difference detected between infection status populations by gender, race, or distance between residence and Hospital Ampang. A significant difference was detected between primary and secondary infections among those aged 45 years and older.

Study participants stratified by dengue infection status were admitted to Hospital Ampang, on average, 4.17 ± 1.75 (primary) and 4.04 ± 1.49 (secondary) days after self-reported onset of illness and had an average length of hospitalization of 5.15 ± 3.93 (primary) and 4.39 ± 2.30 (secondary) days.

Table 2. Study Participants Demographics by Dengue Infection Status and Effusion Status in Hospital Ampang, Selangor, Malaysia 2010.

	Dengue Infection Status				Effusion Status				Total	
	Primary		Secondary		Absence		Presence			
	N	%	*N*	%	*N*	%	*N*	%	*N*	%
Total	41	20.8	156	79.2	115	58.4	82	41.6	197	100
Gender										
Male	25	61.0	99	63.5	77	67.0	47	57.3	124	62.9
Female	16	39.0	57	36.5	38	33.0	35	42.7	73	37.1
Race										
Malay	24	58.5	102	65.4	75	65.2	51	62.2	126	64.0
Chinese	12	29.3	32	20.5	24	20.9	20	24.4	44	22.3
Indian	4	9.8	9	5.8	8	7.0	5	6.1	13	6.6
Other	1	2.4	13	8.3	8	7.0	6	7.3	14	7.11
Age										
15-24	15	36.6	59	37.8	39	33.9	35	42.7	74	37.6
25-34	19	46.3	50	32.1	42	36.5	27	32.9	69	35.0
35-44	5	12.2	23	14.7	17	14.8	11	13.4	28	14.2
>45	2	4.9	24	15.4‡	17	14.8	9	11.0	26	13.2
Distance[&]										
≤ 12.5	33	80.5	127	81.4	95	82.6	65	79.3	160	81.2
> 12.5	8	19.5	29	18.6	20	17.4	17	20.7	37	18.8

P-value: † <0.05; ‡ <0.01

[&] Distance between patient residence and Hospital Ampang.

Stratification of the study population found the majority of participants were free of hemorrhage/vascular leakage manifestations (57.3%) (Table 2). The gender ratio (male: female) was 2.03:1.0 and 1.34:1.0 for absence and presence of hemorrhage/vascular leakage, respectively. Among both study categories of effusion status, the majority of participants were male, Malay, and lived within 12.5 km of Hospital Ampang. Among those individuals with absence of effusion the majority were aged 25-34 years; whereas among those individuals with presence of effusion the majority were aged 15-24 years.

There was no significant difference detected between effusion status populations by gender, race, age, or distance between residence and Hospital Ampang. Study participants stratified by effusion status were admitted to Hospital Ampang, on average, 4.04 ± 1.41 (absence) and 4.10 ± 1.72 (presence) days after self-reported onset of illness and had an average length of hospitalization of 4.67 ± 2.95 (absence) and 4.37 ± 2.39 (presence) days.

Co-Morbidities

Overall, co-morbidities were only observed in 7.6% of the study population (Table 3). Hypertension was the most observed co-morbidity in the population (4.1%), followed by diabetes mellitus (3.1%). One individual was found to have ischemic heart disease. No chronic kidney disease or congestive heart failure was observed.

Table 3. Study Participants Co-Morbidities by Dengue Infection Status and Effusion Status in Hospital Ampang, Selangor, Malaysia 2010.

	Infection Status				Effusion Status				Total	
	Primary		Secondary		Absence		Presence			
	N	%	N	%	N	%	N	%	N	%
Study Participants	41	20.8	156	79.2	115	58.4	82	41.6	197	100
Diabetes mellitus	2	4.9	4	2.6	3	2.6	3	3.7	6	3.1
Hypertension	2	4.9	6	3.9	4	3.5	4	4.9	8	4.1
Other*	0	0.0	1	0.6	1	0.9	0	0.0	1	0.5
Total Diseased	4	9.9	11	6.9	8	7.0	7	8.5	15	7.6

* Chronic kidney disease, congestive heart failure and ischemic heart disease.

Manifestations

Among the study population, the most common manifestations were nausea/vomiting (86.3), arthraglia/myaglia (80.7%), and viral hepatitis (75.6%). The least common manifestations among the study population were rash (16.2%), hemorrhage (19.8%), and hepatosplenomegaly (27.4%). Stratification by dengue infection status or effusion status did not find a significant difference by each manifestation.

Table 4. Study Participants Manifestations by Dengue Infection Status and Effusion Status in Hospital Ampang, Selangor, Malaysia 2010.

	Infection Status				Effusion Status				Total	
	Primary		Secondary		Absence		Presence			
	N	%	N	%	N	%	N	%	N	%
Total Study Participants	41	20.8	156	79.2	115	58.4	82	41.6	197	100
Abdominal Pain	29	70.7	101	64.7	73	63.5	57	69.5	130	66.0
Arthraglia/Myaglia	31	75.6	128	82.1	91	79.1	68	82.9	159	80.7
Diarrhea	21	52.2	89	55.8	68	59.1	42	51.2	110	55.8
Hepatosplenomegaly	9	22.0	45	28.9	31	27.0	23	28.1	54	27.4
Nausea/Vomiting	36	87.8	134	85.9	99	86.1	71	86.6	170	86.3
Postural Giddiness	16	39.0	58	37.2	43	37.4	31	37.8	74	37.6
Rash	8	19.5	24	15.4	20	17.4	12	14.6	32	16.2
Retro-orbital/Headache	14	34.2	79	50.6	57	49.6	36	43.9	93	47.2
Viral Hepatitis	29	70.7	120	76.9	88	76.5	61	74.4	149	75.6
Hemorrhage	10	24.4	29	18.6	-	-	-	-	39	19.8
Vascular Leakage	11	26.8	44	28.2	-	-	-	-	55	27.9

Clinical Laboratory Results

The study population, on average, had an absolute monocyte count of 1.22 ± 0.94 (x10^9/L), platelet count of 56.9 ± 38.4 (x10^9/L), and a hematocrit of 41.6 ± 5.0 percent (Table 5). Absolute monocyte count was observed to be higher in secondary (1.35 ± 0.98) infections compared to primary (0.65 ± 0.37); whereas, platelet count was observed to higher in primary (76.0 ± 38.7) infections compared to secondary (51.9 ± 36.5). Stratification by dengue infection status found a significant difference in absolute monocyte count (p-value= <0.0001) and platelet count (p-value= 0.0002). No significant difference was detected when the study population was stratified by effusion status for absolute monocyte count, platelet count, or hematocrit. An independent sample t-test was employed to determine significance.

Table 5. Clinical Laboratory Manifestations of Study Participants by Dengue Infection Status and Effusion Status in Hospital Ampang, Selangor, Malaysia 2010.

	Infection Status		Effusion Status^		Total
	Primary	Secondary	Absence	Presence	
	Avg. ± Std.	Avg. ± Std.	Avg. ± Std.	Avg. ± Std.	Avg. ± Std.
Absolute Monocyte Count*	0.66 ± 0.37	1.37 ± 0.98 ‡	1.18 ± 0.90	1.28 ± 0.99	1.22 ± 0.94
Platelet Count *	75.6 ± 39.1	52.0 ± 36.8 ‡	55.6 ± 38.0	58.8 ± 39.1	56.9 ± 38.4
Hematocrit (%)	41.1 ± 5.3	41.7 ± 4.9	42.1 ± 4.8	41.0 ± 5.1	41.6 ± 5.0

P-value: † <0.05; ‡ <0.01

* x10^9/liter

Linear Regression Analysis

A multivariable linear regression analysis was used to determine the association of venous absolute monocyte count with primary and secondary dengue infection. A literature review provided the foundation for inclusion of the risk factors gender, race, and age. A surrogate variable was assessed for inclusion in the model for the known risk factors of infecting viral serotype, socioeconomic status, and education. The surrogate variable used was distance of participants' residence from Hospital Ampang dichotomized by 12.5 km.

To determine if the surrogate variable was a potential risk factor for the association stratification of the exposure and outcome by distance of participants' residence from Hospital Ampang was completed. The outcome (independent sample t-test, p-value = <0.001) and the exposure (chi-square, p-value=0.02) were both significantly associated with distance of participants' residence from Hospital Ampang. As a result, distance of participants' residence from Hospital Ampang was considered a risk factor of the association and assessed for inclusion in the regression model.

To assess the assumption that the data is normally distributed, graphical analysis was used to plot the regression model predicted values against the model residuals (Figure 32). Residuals are a measure of variation within the dataset and are calculated by determining the difference between the observed and expected values. A perfect fit of the model to the data would result in all

residuals being zero; however, this is almost never observed. Under a hypothetical normal distribution, a plot of the model predicted values against model residuals will result in equal variance above and below zero. Graphical analysis of the model predicted values against the model residuals of the dataset under the base model displays a distribution that approaches normality. The determination that the normality is approximated suggests the correct use of the linear regression model for the analysis.

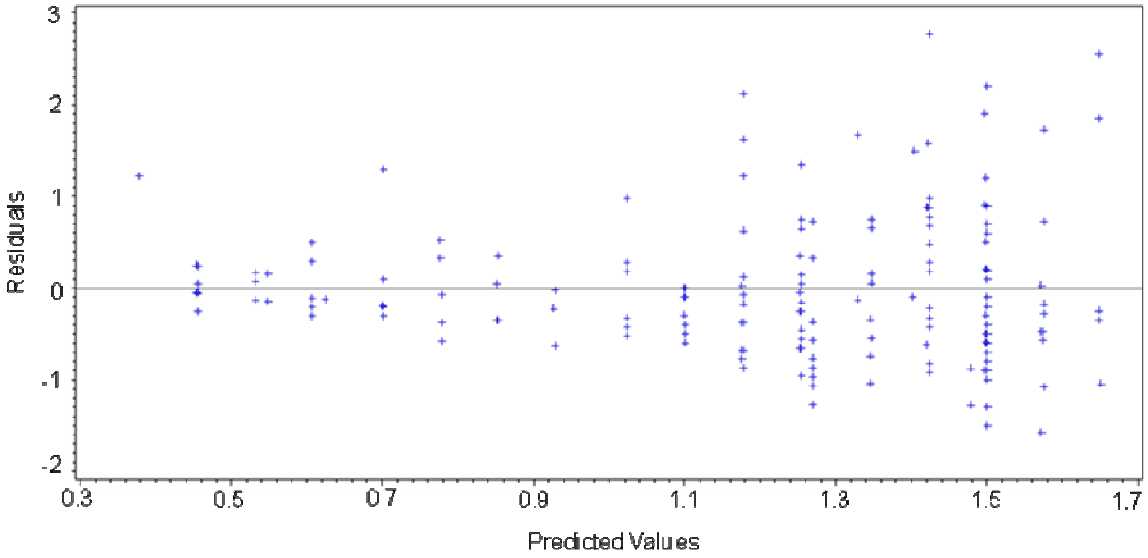


Figure 32. Scatter plot of residuals versus the predicted values of venous absolute monocyte levels based on linear regression model with age as categorical variable and without the distance variable.

The regression coefficient (estimate), coefficient standard error, and p-values are presented in Table 6 for the above regression model. Regression

coefficients are estimates of the independent contribution of each independent variable to the prediction of the dependent variable, otherwise known as partial correlation. Interpretation of the coefficients is first completed by observing the signs (plus/minus). A positive coefficient delineates a positive relationship (as the independent variable increases, so does the dependent variable) of the independent variable with the dependent variable. Whereas, a negative coefficient means the independent variable and dependent variable have a negative relationship (when the independent variable increases, the dependent variable decreases). A coefficient of zero infers no relationship between the independent variable and dependent variable.

The value of the coefficient for each of the independent variables is a measure of the effect of that particular variable has on the dependent variable. In multivariable linear regression models, the coefficient is how much the dependent variable is expected to increase when the independent variable is increased by one, while holding all other independent variables constant.

Under the base model, dengue infection status (p-value= <0.0001) was independently associated with venous absolute monocyte levels. All other independent variables did not demonstrate a significant independent association with venous absolute monocyte levels.

Table 6. Estimates from Linear Regression Model of Venous Absolute Monocyte Levels during Defervescence controlling for Dengue Infection Status, Age (categorical), Gender, and Race.

Independent Variable	Estimate	Standard Error	p-value
Intercept	-0.19	0.33	0.57
Infection Status	0.72	0.16	<0.0001
Age	-0.08	0.06	0.22
Gender	0.25	0.13	0.06
Race	0.08	0.07	0.29

The coefficient of determination (R-squared) is a measure of the how much of the data variance is explained by the linear model, or in other words, how well the model fits the data. The coefficient of determination for the base model is 0.127, meaning the model explains 12.7% of the variability in the dataset.

The distance of the participants' residence from Hospital Ampang was added to the above model to assess any influence on the association of interest. Graphical analysis using a plot of model predicted values against residuals determined that under the model, the distribution approached normality suggesting correct use of linear regression for the analysis (Figure 33).

A negative distance coefficient ($\beta = -0.40 \pm 0.16$, p-value= 0.01) infers that greater distance from Hospital Ampang is significantly associated with lower monocyte levels (Table 7). Infection status has a positive coefficient ($\beta = 0.72 \pm 0.15$, p-value= <0.0001), meaning that a secondary infection is significantly

associated with increased monocyte count. A positive gender coefficient (β= 0.26 ± 0.13, p-value= 0.05) infers that male gender is significantly associated with higher monocyte count. Age (β= -0.09 ± -0.06, p-value= 0.14) and race (β= 0.05 ± 0.07, p-value= 0.5) did not have a significant effect on the dependent variable.

The addition of distance from the participants' residence to Hospital Ampang improved the linear fit of the model to the data, explaining 15% of the dataset variability (r-squared = 0.15).

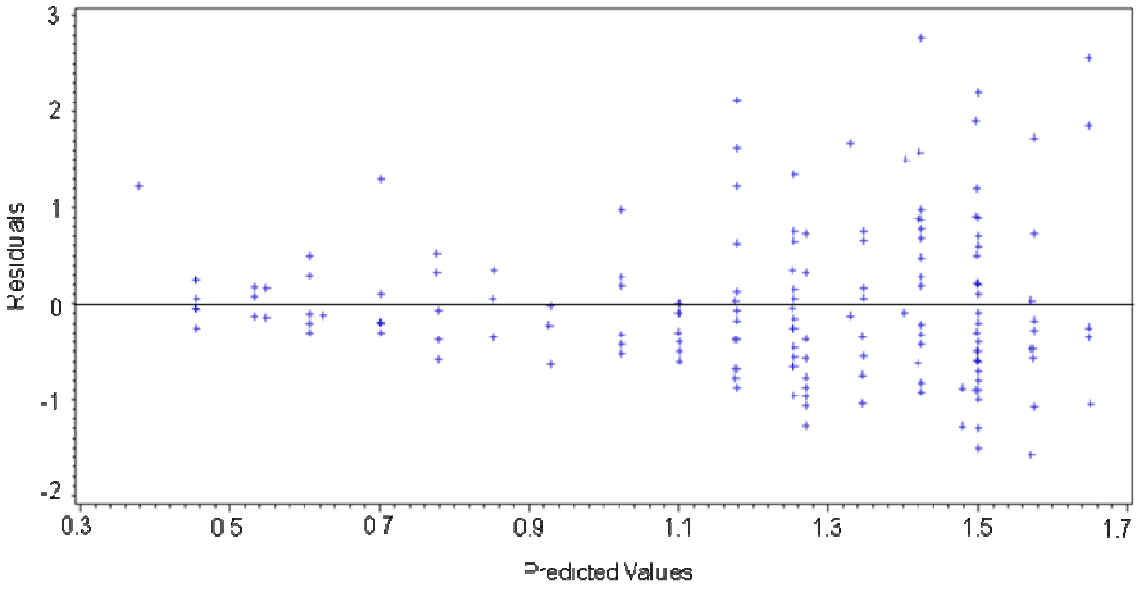


Figure 33. Scatter plot of residuals versus the predicted values of venous absolute monocyte levels based on linear regression model with age as categorical variable and including distance variable.

Table 7. Estimates from Linear Regression Model of Venous Absolute Monocyte Levels during Defervescence controlling for Dengue Infection Status, Age (categorical), Gender, Race, and Distance between Patient Residence and Hospital Ampang.

Independent Variable	Estimate	Standard Error	P-value
Intercept	-0.05	0.33	0.89
Infection Status	0.72	0.15	<0.0001
Age	-0.09	-0.06	0.14
Gender	0.26	0.13	0.05
Race	0.05	0.07	0.49
Distance	-0.40	0.16	0.01

To determine if the age variable cut-points were masking or creating a true association, the model was re-assessed with the age as a continuous variable. A model not including the distance variable was initially fit. Graphical analysis using a plot of model predicted values against residuals determined that under the model, the distribution approached normality suggesting correct use of linear regression for the analysis (Figure 34).

Infection status has a positive coefficient (β= 0.72 ± 0.16, p-value= <0.0001), meaning that a secondary infection is significantly associated with increased monocyte count (Table 8). Gender (β= 0.25 ± 0.13, p-value= 0.06), age (β= -0.09 ± -0.06, p-value= 0.14), and race (β= 0.05 ± 0.07, p-value= 0.5) did not have a significant effect on the dependent variable.

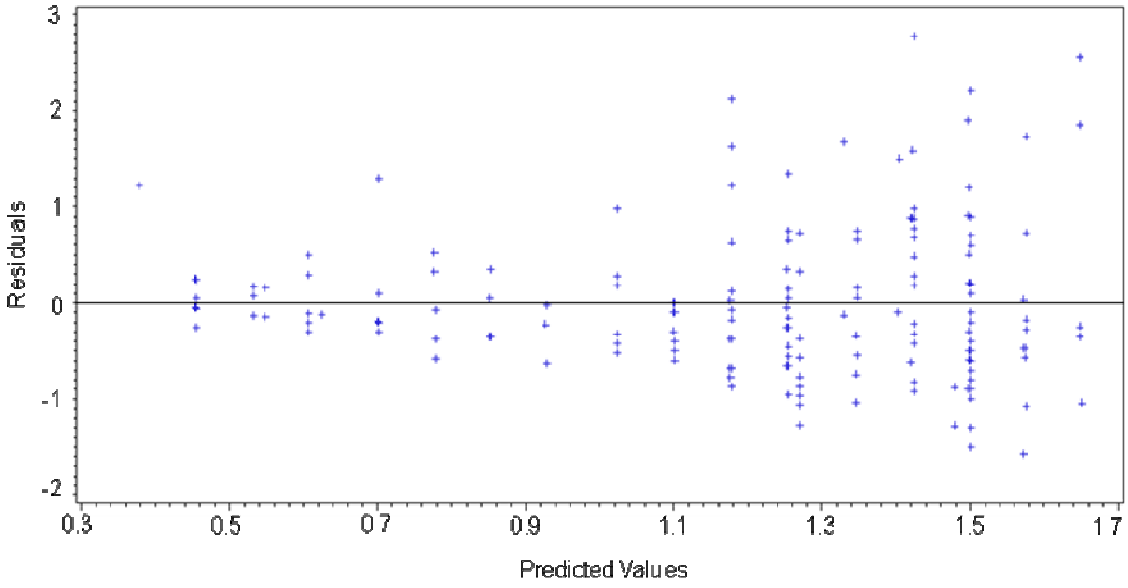


Figure 34. Scatter plot of residuals versus the predicted values of venous absolute monocyte levels based on linear regression model with age as continuous variable and without distance variable.

Table 8. Estimates from Linear Regression Model of Venous Absolute Monocyte Levels during Defervescence controlling for Dengue Infection Status, Age (continuous), Gender, and Race.

Independent Variable	Estimate	Standard Error	p-value
Intercept	-0.07	0.34	0.84
Infection Status	0.71	0.15	<0.0001
Age	-0.01	0.01	0.08
Gender	0.25	0.13	0.06
Race	0.08	0.07	0.24

Assessing the age as a continuous variable did have an impact on the model in that gender became non-significant and the r-square value was 0.13.

The distance of the participants' residence from Hospital Ampang was added to the above model to assess the variable's influence on the association of interest. Graphical analysis using a plot of model predicted values against residuals determined that under the model, the distribution approached normality suggesting correct use of linear regression for the analysis (Figure 35).

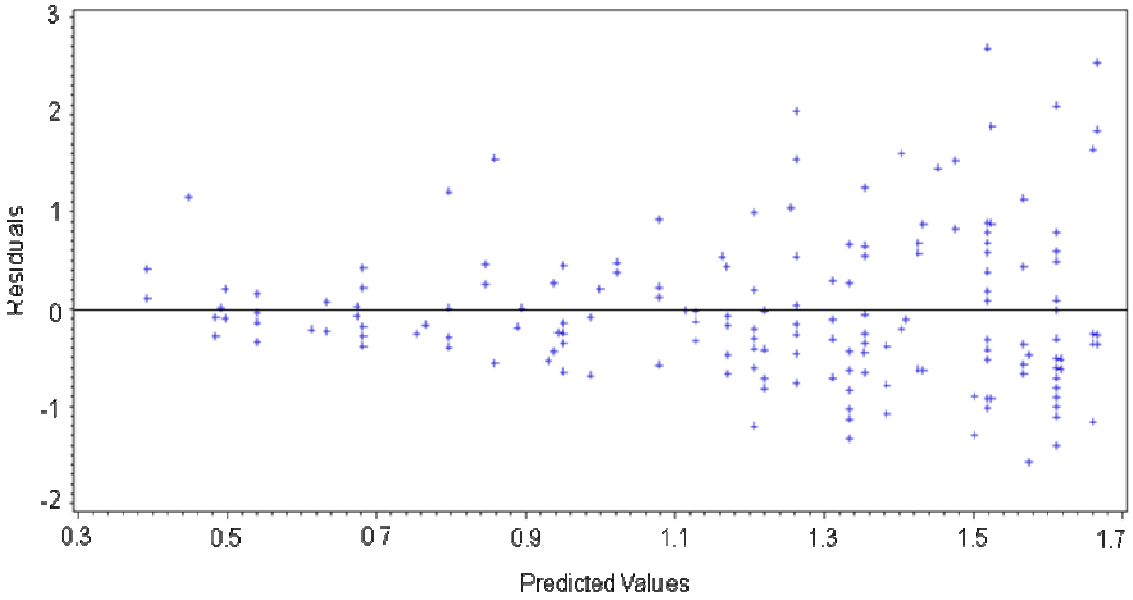


Figure 35. Scatter plot of residuals versus the predicted values of venous absolute monocyte levels based on linear regression model with age as continuous variable and including distance variable.

A negative distance coefficient (β= -0.40 ± 0.06, p-value= 0.02) infers that greater distance from Hospital Ampang is significantly associated with lower monocyte levels (Table 7). Infection status has a positive coefficient (β= 0.71 ± 0.15, p-value= <0.0001), meaning that a secondary infection is significantly associated with increased monocyte count. A positive gender coefficient (β= 0.26 ± 0.13, p-value= 0.04) infers that male gender is significantly associated with higher monocyte count. Age (β= -0.01 ± -0.01, p-value= 0.06) and race (β= 0.06 ± 0.07, p-value= 0.4) did not have a significant effect on the dependent variable.

The addition of distance from the participants' residence to Hospital Ampang improved the linear fit of the model to the data, explaining 16% of the dataset variability (r-squared = 0.16).

Table 9. Estimates from Linear Regression Model of Venous Absolute Monocyte Levels during Defervescence controlling for Dengue Infection Status, Age (continuous), Gender, Race, and Distance between Patient Residence and Hospital Ampang.

Independent Variable	Estimate	Standard Error	P-value
Intercept	0.05	0.34	0.87
Infection Status	0.71	0.15	<0.0001
Age	-0.01	0.01	0.06
Gender	0.26	0.13	0.04
Race	0.06	0.07	0.41
Distance	-0.37	0.16	0.02

Overall, including the distance surrogate variable and using age as a continuous variable improved the linear fit of the model. For this investigation, estimates from the model with age as a continuous variable and including distance in the model will be accepted as the final model. However, future investigations should strive to capture infecting viral serotype, socioeconomic status, and education data to include in the model in place of the surrogate variable.

Logistic Regression Analysis

A multivariable logistic regression model was used to determine the association between venous absolute monocyte levels and effusion status (presence or absence of hemorrhage/vascular leakage). A literature review provided the foundation for inclusion of gender, race, age, and dengue infection status (primary or secondary) in the initial model. Age was initially assessed as a categorical variable. Assessment of distance of participants' residence from Hospital Ampang reveled an association with the exposure (independent sample t-test, p-value = <0.001); however, no association was observed with the outcome (chi-square, p-value= 0.35). While the criteria to be a confounder for the association was not met, distance of participants' residence from Hospital Ampang was assessed in the analysis due to the need to control for other known risk factors (infecting viral serotype, socioeconomic status, and education).

Unlike linear regression models, logistic regression modeling does not make assumptions about the distribution of the independent variables; however, testing the fit of the model is important to avoid spurious results. To determine if the fit of the base model was adequate, graphical analysis was used plotting model Pearson residuals against estimated probability (Figure 36). If a model is adequately fit, an approximately horizontal line will connect the end points in the plot and have an intercept of zero. As a deviation from these criteria was observed in the base model graphical analysis, this suggests the model is inadequate to predict the outcome.

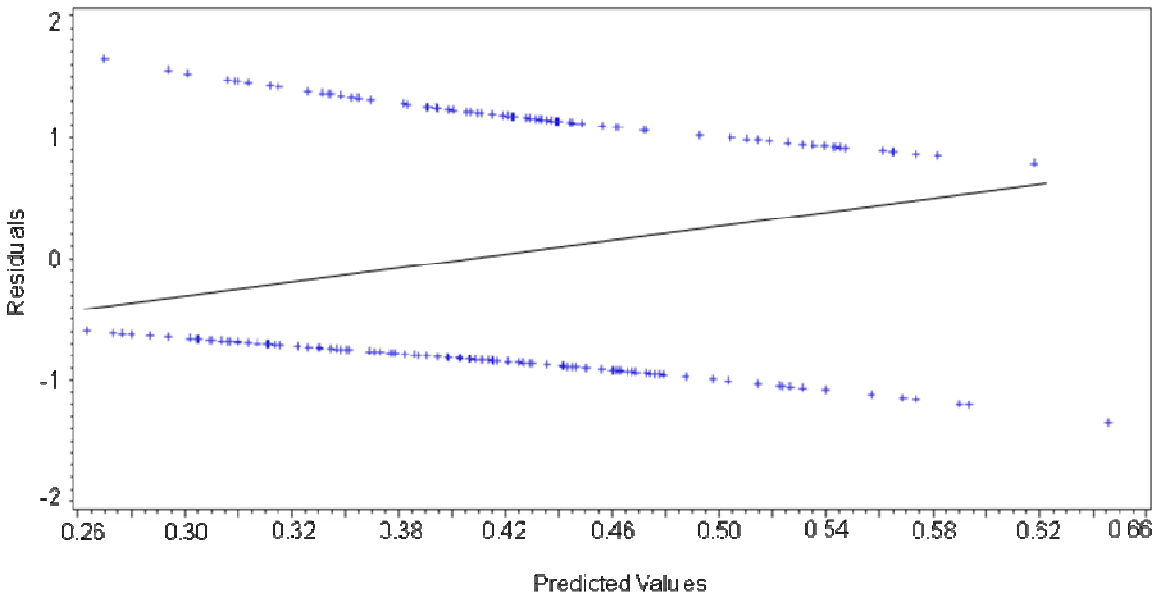


Figure 36. Scatter plot of Pearson residuals versus the predicted values of venous absolute monocyte levels based on logistic regression model with age as categorical variable.

All independent variables under investigation did not display an independent relationship with the dependent variable (absence and presence of hemorrhage/vascular leakage) (Table 10).

The Hosmer-Lemshow test for goodness-of-fit is a chi-square statistic testing whether or not the predicted model significantly differs from the observed data; the desired outcome of the test is non-significance. The Hosmer-Lemshow test for goodness-of-fit for the base model is non-significant (χ^2= 0.26) meaning that model accurately models the observed data.

Table 10. Estimates from Logistic Regression of Effusion Status Controlling for Venous Absolute Monocyte Count, Age (categorical), Gender, Race, and Dengue Infection Status.

Independent Variable	Reference		Estimate	Standard Error	P-value
Intercept			-0.53	0.37	0.15
Monocyte			0.17	0.17	0.33
Age	15-24	>45	-0.50	0.49	0.31
		35-44	-0.41	0.46	0.38
		25-34	-0.39	0.35	0.26
Gender	Male		0.47	0.31	0.13
Race	Malay	Other	0.13	0.59	0.82
Race		Indian	0.05	0.62	0.93
Race		Chinese	0.16	0.37	0.67
Infection Status	Secondary		0.08	0.40	0.83
Hosmer and Lemeshow Goodness of fit = χ^2 = 0.26					

To determine if distance of participants' residence from Hospital Ampang affects the relationship of interest, the variable was added to the above model. Graphical analysis of the logistic regression model plotting Pearson residuals against estimated probability determined that the model was inadequate to predict the outcome (Figure 37). All independent variables under investigation did not display an independent relationship with the dependent variable (Table 11). The Hosmer-Lemshow test for goodness-of-fit for the base model is non-significant (χ^2= 0.07) meaning that model accurately models the observed data.

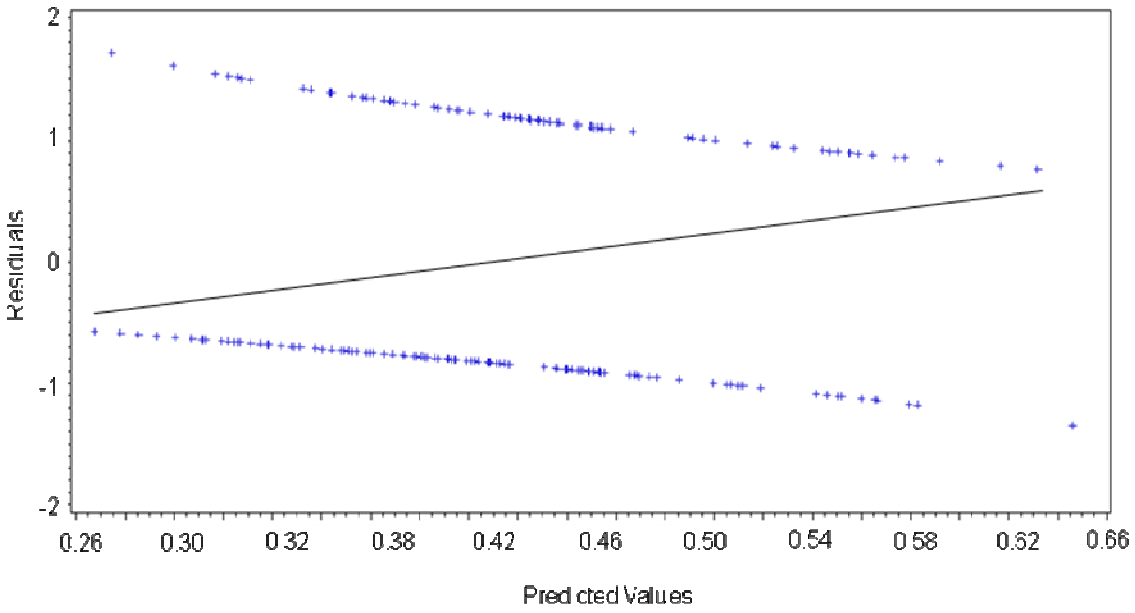


Figure 37. Scatter plot of Pearson residuals versus the predicted values of venous absolute monocyte levels based on logistic regression model with age as categorical variable and including distance variable.

Table 11. Estimates from Logistic Regression of Effusion Status Controlling for Venous Absolute Monocyte Count, Age (categorical), Gender, Race, and Dengue Infection Status.

Independent Variable	Reference		Estimate	Standard Error	P-value
Intercept			-0.65	0.41	0.11
Monocyte			0.19	0.18	0.28
Age	15-24	>45	-0.47	0.49	0.34
		35-44	-0.39	0.46	0.40
		25-34	-0.36	0.35	0.31
Gender	Male		0.48	0.31	0.13
Race	Malay	Other	0.59	0.59	0.79
Race		Indian	0.62	0.62	0.87
Race		Chinese	0.37	0.37	0.58
Infection Status	Secondary		0.09	0.40	0.82
Distance	≤12.5km		0.28	0.39	0.48
Hosmer and Lemeshow Goodness of fit = $\chi^2 = 0.07$					

To determine if the age variable cut-points were masking or creating a spurious association, the model was re-assessed with the age as a continuous variable. A model without the distance variable was initially assessed. Graphical analysis of the logistic regression model plotting Pearson residuals against estimated probability determined that the model was inadequate to predict the outcome (Figure 38). All independent variables under investigation did not display an independent relationship with the dependent variable (Table 12). The Hosmer-Lemshow test for goodness-of-fit for the base model is non-significant ($\chi^2 = 0.64$) meaning that model accurately models the observed data.

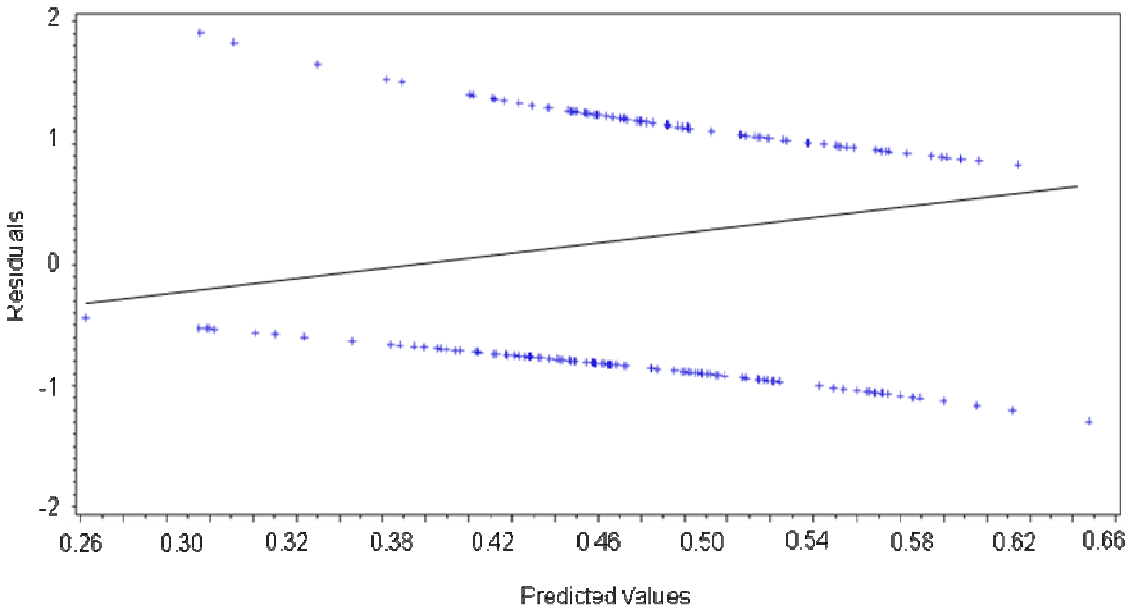


Figure 38. Scatter plot of Pearson residuals versus the predicted values of venous absolute monocyte levels based on logistic regression model with age as continuous variable and without the distance variable.

Table 12. Estimates from Logistic Regression of Effusion Status Controlling for Venous Absolute Monocyte Count, Age (continuous), Gender, Race, and Dengue Infection Status.

Independent Variable	Reference		Estimate	Standard Error	P-value
Intercept			-0.06	0.53	0.91
Monocyte			0.11	0.17	0.52
Age			-0.02	0.01	0.13
Gender	Male		0.48	0.31	0.12
Race	Malay	Other	0.10	0.58	0.87
Race		Indian	0.08	0.62	0.90
Race		Chinese	0.18	0.36	0.62
Infection Status	Secondary		0.03	0.38	0.95
Hosmer and Lemeshow Goodness of fit = $\chi^2 = 0.64$					

To determine if distance of participants' residence from Hospital Ampang affects the relationship of interest, the variable was added to the above model. Graphical analysis of the logistic regression model plotting Pearson residuals against estimated probability determined that the model was inadequate to predict the outcome (Figure 39). All independent variables under investigation did not display an independent relationship with the dependent variable (Table 11). The Hosmer-Lemshow test for goodness-of-fit for the model is non-significant (χ^2= 0.59) meaning that model accurately models the observed data.

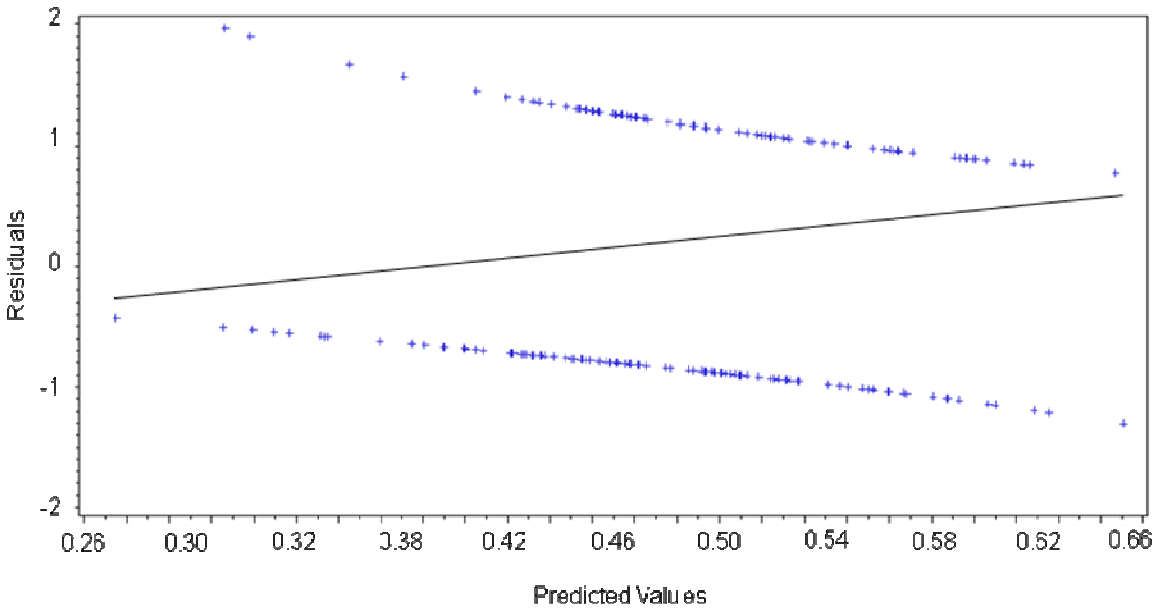


Figure 39. Scatter plot of Pearson residuals versus the predicted values of venous absolute monocyte levels based on logistic regression model with age as continuous variable and including distance variable.

Table 13. Estimates from Logistic Regression of Effusion Status Controlling for Venous Absolute Monocyte Count, Age (continuous), Gender, Race, Dengue Infection Status, and Distance between Patient Residence and Hospital Ampang.

Independent Variable	Reference		Estimate	Standard Error	P-value
Intercept			-0.17	0.56	0.76
Monocyte			0.13	0.17	0.46
Age			-0.02	0.01	0.14
Gender	Male		0.49	0.31	0.12
Race	Malay	Other	0.12	0.58	0.84
Race		Indian	0.11	0.62	0.85
Race		Chinese	0.22	0.36	0.54
Infection Status	Secondary		0.04	0.38	0.92
Distance	≤12.5km		0.26	0.39	0.50
Hosmer and Lemeshow Goodness of fit = $\chi^2 = 0.59$					

Overall, the inability of the model building process to produce significant findings speaks to the complex biological nature of dengue disease. No acceptable final model was produced in this research for the secondary objective as all models were found inadequate to predict the outcome. As researchers continue to unravel the complex dengue disease biological pathway future attempts to model disease outcome by including new variables might have better success.

DISCUSSION

Prior to the discussion of the study results, an assertion of the internal validity of the study is necessary. Internal validity of a study is based around the premise of a high degree of precision in the association under investigation (Oleckno, 2002). If a study does not maintain internal validity, external validity cannot be achieved. In epidemiologic studies, three factors can affect internal validity: chance, bias, and confounding.

Chance, also known as random error, can be separated into two primary sources: measurement and sampling error. Measurement error occurs during the measurement of individual values for study analysis and is avoided through diligent study planning. The two sources of data for the study were clinical data from Hospital Ampang and laboratory data from the Medical Microbiology Laboratory of Professor Dr. Shamala Devi. All clinical data from Hospital Ampang was collected from the hospital electronic database. Only trained medical professionals have access and can enter values into the hospital electronic database, providing a high degree of certainty about the measurements. Both laboratories of Hospital Ampang and Professor Dr. Shamala Devi's employed the use of standardized controls. These controls

allowed the trained laboratory personnel to determine if the assay was working properly, reducing measurement error.

Sampling error is the "variation that can result when using sampling statistics to estimate population parameters" (Oleckno, 2002, p.152). Sampling error is reduced by having a large enough study population to detect the association of interest above any random variation. While one may haphazardly overestimate the study population needed, an efficient use of both time and limited financial resources is achieved through *a priori* sample size calculation.

As the study was subset within another study, an *a priori* sample size calculation was not feasible. A retrospective power calculation conducted based on literature estimates ensures that if an association did exist, it could be detect above variations within sampling. The retrospective power calculation determined that the study had a power of 0.87, meaning that if an association exists, it will be detected 87% of the time. With studies typically aiming for a power of 0.80 (Oleckno, 2002), the study exceeds standards set for study power.

Bias is "nonrandom error in the design, conduct, or analysis" of an epidemiological study (Oleckno, 2002, p.125) and can be classified as selection or measurement. As bias cannot be addressed after the data has been collected, careful study design planning is necessary to avoid spurious results.

Selection bias is the nonrandom error by which study participants are selected or retained (Oleckno, 2002). To avoid selection bias, collection of

subjects must be conducted so that the study population represents the target population, subject loses are kept to a minimum, and comparison groups within the study are similar except for the variables being investigated (Oleckno, 2002).

Data on the target population of the study were not available; however, summary statistics on the approximate (in- and out-patient) source population were obtained for the 2010 calendar year. No significant difference was detected between the approximate source population, source study population, and study population by age, gender, and race.

Successful participant recruitment and reduction of loss to follow-up is critical to minimizing bias within the study. With participation rate slightly above the standard cut-off of 20% (Galea & Tracy, 2007) and retention rate of 96.2%, the source study design was successfully executed, reducing a major source of bias. To confirm this statement, chi-squared tests for independence were conducted between the source study population and the population of individuals who refused to participate by gender, race, age, and distance between residence and Hospital Ampang; no significant difference was observed. The finding of no difference between populations infers that recruitment bias had negligible impact on the study, meaning patients decided to participate in the study in a non-systematic way.

Ideally, a study will recruit a population which does not significantly differ by risk factors so that the association of interest can be correctly assessed.

When the study population was stratified by the exposure of interest, primary and secondary dengue infection, there was no significant difference detected by gender, race, or distance between residence and Hospital Ampang. However, a significant difference was detected between primary and secondary infections among those aged 45 years and older. This finding is understandable in that as a person ages they will have been at increased risk for multiple dengue infections compared to an individual of younger age while living in a dengue endemic environment. Also, as an individual ages their immune system is less capable of handling disease stress necessitating more medical intervention compared to young populations. The use of multivariable analysis will allow for consideration of this difference to correctly assess the association of interest. The above findings provide assurance that negligible amounts of selection bias were introduced into the study as far as can be detected.

Measurement bias is the "nonrandom error in classifying subjects with regard to exposure or outcome status" that may result from inaccurate measurements (Oleckno, 2002, p.144). As both the exposure and outcome of interest were determined through the use of standardized laboratory assays, both of which employed controls, the risk for differential misclassification is minimal. If the assay standardized controls were not met, enough of the venous serum sample remained to repeat the assay several times until control verification was met.

Confounding is nonrandom error by the distortion of the association of interest by an incidental factor. A confounder, termed risk factor in epidemiology, must be 1) associated with the exposure; 2) an independent risk factor for the outcome; 3) must not be an intermediate variable on the causal pathway; and 4) must be present in different degrees between study groups (Oleckno, 2002). Confounding can be controlled during the planning phase of the study through restriction, matching, and/or randomization. Multivariable analysis during the analysis phase of the study can control for confounding effects only if they were measured during the study (Oleckno, 2002).

Potential risk factors of the primary relationship of interest include gender, race, and age, which were determined through the literature review. Knowledge of the study location suggested distance between patient residence and Hospital Ampang might be a potential risk factor. Stratification of the exposure and outcome by the risk factor allowed for associations to be assessed. As there was an association of the risk factor with the exposure and outcome, distance between the participants residence with Hospital Ampang was classified as a potential risk factor for the primary relationship of interest. All risk factors were taken into account in the multivariable analysis.

Having successfully addressed, as far as can be determined, the potential effects of chance, bias, and confounding on the study, the study internal validity has been maintained. The following presents a discussion of the study

population disease duration, co-morbidities, manifestations, and clinical laboratory results with comparison to findings from the literature.

The duration of time from onset of illness to hospital admission and length of hospitalization was similar across the source study and study populations. From the literature, a 2008 cross-sectional study among 236 dengue patients from two hospitals in Federal Territory of Kuala Lumpur and State of Selangor, Malaysia, found the average length of time from onset of illness to admission to be 4.9 days (Ang, Rohani, & Look, 2010). A separate prospective cohort study of 77 dengue hospitalized adults in Kuala Lumpur, Malaysia, from December 2004 to December 2005, observed the average length of hospitalization to be 2.4 ± 5.3 days (Lum *et al.*, 2008). While the study population and literature findings had similar durations between onset of illness and admission, the length of hospitalization was longer in the study population compared to the literature. The dissimilar findings might be due to differences in severity of dengue disease within the populations and/or standard of care between hospitals. There are no exact criteria for patient discharge and is highly dependent on overall patient health. Further research would be need to elucidate why discharge time periods differ.

The low prevalence of co-morbidities (7.6%) within the study population is potentially reflective of the relative young overall age of study participants (31.2 ± 11.7 years). Comparable proportions of co-morbidities in the study population, when stratified by dengue infection and effusion status, indicates a comparatively

homogenous study population with respect to diagnosed chronic diseases. From the literature, a study of 120 dengue patients aged 15 to 73 years in Singapore from 2000-2005 indicated a similar prevalence of co-morbidities within the study population (10%) (Lye, Chan, Lee, & Leo, 2008). The low prevalence of co-morbidities in both the study population and findings from literature, allow for the observation that dengue is affecting a mostly healthy population and inference that co-morbidities might not have a significant role in the development of severe dengue complications. While not observed in the study population, co-morbidities that have been associated with adverse dengue outcomes include obesity, diabetes mellitus, hypertension and chronic heart diseases (Farrar *et al.,* 2007).

Dengue disease commonly manifests with rash, nausea/vomiting, and arthraglia/myaglia (WHO/TDR, 2009). Study participants were observed in high proportions to have nausea/vomiting and arthraglia/myaglia; however, rash was the least observed manifestation. The low presence of rash within the study population may be due to the overall late admission (~day 4 of illness) to Hospital Ampang by which time rash may have resolved. Further dissimilarities between the study population and literature from Kuala Lumpur (Lum *et al.,* 2008), include the low proportion of study participants manifesting with pleural effusion, bleeding, dizziness, and abdominal pain. A review of dengue literature outside Malaysia has found populations with similar proportional manifestations to that of the study population (Thomas *et al.,* 2010; Kumar *et al.,* 2010). The differences

and similarities in manifestations among the study population compared to findings in the literature, illustrates the high degree of variation in dengue disease clinical manifestations. Clinicians must be aware of the wide-range of manifestations that can be observed during dengue disease. Using the new dengue classification criteria proposed by WHO in 2009, all study participants were capable of being classified. This finding supports the use of new classification criteria; however, future studies are needed to ensure continued flexibility of the classification criteria, especially as populations in dengue endemic counties begin to age.

Analysis of each manifestation by both primary & secondary dengue infection and absence & presence of hemorrhage/vascular leakage did not observe a significant difference. These findings are not unexpected as the study population is comprised of patients who were admitted to Hospital Ampang for severe dengue complications. What is striking is the similarity between primary and secondary for presence of hemorrhage and vascular leakage. Commonly, hemorrhage and vascular leakage are thought of as complications only observed in heterotypic secondary dengue infections. The findings are a reminder that severe dengue complications are not just manifestations that occur in secondary heterotypic dengue infections. However, the similar observed proportions of hemorrhage and vascular leakage in the study population are not representative of those experienced in the general population, as the study was designed to focus on those individuals who develop severe complications. Among the

general population, the majority of primary dengue infections will not develop severe complications, whereas those experiencing a secondary infection will be at an increased risk for severe complications.

The wide range of manifestations in dengue disease highlights the need for a highly flexible dengue diagnostic scheme to clinically classify dengue patients. A recent review in 18 countries shows that the 2009 dengue diagnostic scheme could classify all but 1.7% of study participants; whereas the 1997 dengue diagnostic classification criteria could not classify 13.7% of study participants (Barniol *et al.,* 2011). There is still a need for further research and possible re-structuring of the dengue classification criteria to ensure that all individuals who are laboratory diagnosed with dengue can be readily diagnosed.

A decreasing platelet count and concurrent increasing hematocrit, are early clinical warning signs of potentially severe dengue complications (WHO/TDR, 2009). The study population mean hematocrit remained within the normal range of 36.0-48.0%, most likely as a result of the immediate and attentive rehydration therapy administered by the Hospital Ampang medical staff. As more medical facilities increase their rehydration therapy capabilities, hematocrit may not remain a commonly observed indicator of potential severe dengue complications. Platelet count of the study population was well below the 150-400 (x10^9/L) platelet count normal range. As the majority of study patients were even below the 100 (x10^9) platelet count threshold, this gives more

evidence for the appropriate lowering of the platelet count dengue criterion (Rigau-Perez, 2006).

The study population average absolute monocyte count was above the normal range of 0.1 - 0.8 (x10^9/L). However, when the study population was stratified by infection status, the primary infection population average absolute monocyte count reduced back into the normal range, whereas the secondary infection population remained above the normal range. The difference between the primary and secondary infection status absolute monocyte counts was found to be significant. The literature review did not find a study which assessed a similar research relationship. To fully analyze this association, potential risk factors of the association were assessed with multivariable linear regression analysis.

An initial linear regression model was constructed with dependent variable absolute monocyte count and independent variables infection status (primary or secondary), race, age, and gender. Assumptions of the methodology were graphically assessed and fit of the model determined. Distance of the participants' residence from Hospital Ampang was added to the initial model to determine if the variable influenced the association. In the initial model, the age variable was assessed as categorical variable with cut-points used from the literature; however, it was necessary to determine if categorizing the age variable was masking or creating artificial findings. The age variable was run in the initial model as a continuous variable. The distance variable was again added to the

initial model, with a continuous age variable, for reassessment of the influence on the association. Comparing the assumptions of the methodology and fit of the model between the four models, it was determined that the final model would be the model using a continuous age variable and including the distance variable as the final model (Table 9).

The final model finding that secondary dengue infection significantly increases absolute monocyte during defervescence is presumably a novel contribution to the dengue literature. Specifically, a secondary dengue infection increases the venous absolute monocyte count by 0.71±0.15 (x10^9/L) compared to a primary dengue infection, while holding all other independent risk factors constant. Among all independent variables considered in the model, infection status had the largest effect size and was most significant for the association.

As the normal range of venous absolute monocyte count in adults is 0.1 to 0.8 (x10^9/L), the average value observed in those with primary dengue infection is within these normal bounds; however, secondary dengue infections have an average abnormal elevated count. The severe dengue biological pathway hypotheses of antibody-independent enhancement and memory T-cell reactivation, revolve around the central idea that a previous dengue infection alters the immune response. As a result of the immune system alteration, during a secondary heterotypic dengue infection, severe dengue complications occur. The study does not validate either severe dengue hypothesis, but does provide

further evidence that monocytes are more abundant in a secondary heterotypic dengue immune response compared to a primary dengue immune response. Increased monocyte count is commonly observed in inflammatory disorders (Moore & Tabas, 2011), which supports the biological pathway of severe dengue complications potentially being caused by proinflammatory cytokines released by monocytes. This information will prove valuable in formulating further study hypotheses that may eventually lead to a comprehensive understanding of the severe dengue biological pathway.

Furthermore, the study presents evidence to that while race and age do not significantly influence the relationship better absolute monocyte count and primary & secondary dengue infection, gender and distance of residence from Hospital Ampang do modify the association.

The literature has found an increased risk of severe dengue in children compared to adults and suggests stress from the dengue disease on undeveloped capillaries to be a potential cause of the finding. The study has observed a -0.01 ± 0.01 (x10^9/L) (p-value= 0.06) decrease in absolute monocyte count for each year a person ages, holding all other variables constant. This finding, while non-significant, still remains important as it suggests another potential influence on the association for increased risk in younger aged individuals. The immune system, while typically vibrant and strong in youth, weakens with age. A youthful immune system may lead to an unnecessarily strong immune response causing damage to the capillaries.

The finding also raises the question about what happens in old age when the immune system has weakened so much that it cannot handle the dengue infection. Few studies have investigated the effects of dengue on the elderly. More studies should be conducted on the elderly demographic as dengue is currently spreading to non-endemic regions where the vast majority of the elderly population has not experienced a dengue infection before.

Literature has suggested that genetics (i.e. HLA types) may influence the outcome of a dengue infection. As genetic analysis was not available for inclusion in the study, race was used as a surrogate variable. The analysis determined that race had negligible effect on the primary association of interest (0.06 ± 0.07 x10^9/L, p-value = 0.41), suggesting that race is an imperfect surrogate variable for genetic variation within the study population. Research is needed to identify and confirm genes that increase the risk for severe dengue complications for proper consideration of the potential risk factor.

Blood profiles have differing normal ranges for males and females on many components (e.g. hematocrit & hemoglobin); however, no such difference in normal range by gender exists for absolute monocyte count. Among the study population, an individual of male gender increases the venous absolute monocyte count by 0.26 ± 0.13 (x10^9/L) (p-value=0.04) compared to an individual of female gender, while holding all other independent risk factors constant. The significant influence of male gender on absolute monocyte count,

suggests physiological and/or genetic differences may play a role in the immune response.

From knowledge of the study site, a concern developed that since some patients were being transferred to Hospital Ampang, they would differ from those individuals who go to Hospital Ampang because it was the closest location for medical treatment. All four dengue serotypes are currently co-circulating within Malaysia. Individuals who were being transferred to Hospital Ampang may have contracted a different serotype or strain of dengue virus, which may cause a different immunological response than the predominant virus being circulated near Hospital Ampang. As dengue viremia typically drops below detectable levels by Day 4 of symptomatic illness, and study participants were admitted on average to Hospital Ampang on Day 4.07 ± 1.55 of symptomatic illness, most study participants infecting dengue serotype could not be determined. Only 17 of 197 study participant's dengue infecting serotype were determined through PCR analysis.

Education and socioeconomic status are risk factors for increased incidence of dengue infection. Individuals with higher education levels show protection against dengue infection for several potential reasons. First, they are more likely to have been taught the dangers of dengue and how to prevent infection. Secondly, individuals with higher education tend to earn more which allows them to move to a residence that may provide better protection from the dengue vectors. Lastly, with increased socioeconomic status a family can afford

to take preventative steps to reduce vector breeding sites and can afford privatized medical care that may have better individualized care.

As information on the above factors was not available or incomplete for the entire study population, the average distance (12.5 km) between Hospital Ampang and other public hospitals was used as a generalized surrogate variable. This cut-point was arrived at by averaging the distance between Hospital Ampang and the five nearest public hospitals. This cut-point provides a generalized boundary by which to identify those individuals who travel a further distance to receive care at Hospital Ampang, and those individuals who are travelling to the nearest public hospital.

Among the study population, an individual with a residence of greater than 12.5 km from Hospital Ampang is found to decrease the venous absolute monocyte count 0.37 ± 0.16 (x10^9/L) compared to an individual with a residence equal or less than 12.5 km from Hospital Ampang, while holding all other independent risk factors constant. The significant finding (*p*-value= 0.02) suggests that the study population differed on one of the above mentioned factors. Future studies should include these three risk factors to better consider confounding effects of the relationship of interest.

The analysis of the relationship between absolute monocyte count and primary & secondary dengue infection status considering the effects of independent risk factors has answered the second primary aim. However, after

reviewing the results of the study, more questions have been raised. Hypothetically, if the study results had observed an elevated absolute monocyte count both in primary and secondary dengue infections, one may hypothesize that the elevated monocyte levels cause an increased release of proinflammatory cytokines. The elevated levels of cytokines could then cause the study participant's severe dengue manifestations. While all study participants had severe dengue complications, the absolute monocyte count was significantly increased in secondary dengue infections compared to primary infections. The foremost question that comes out of this finding is what is the biological pathway behind primary dengue infection development of severe dengue complications? As dengue virus can infect monocytes, and monocytes have been shown to release proinflammatory cytokines, normal levels of monocytes in primary dengue infections does not sequentially lead to severe complication development with current dengue knowledge. So, are there multiple biological pathways by which severe dengue manifests? Even with a lower number of monocytes, do severe cases of dengue release similar levels of cytokines? Do monocytes in primary and secondary dengue infection cases release the same type of cytokines? These are the questions which researchers may be able to immediately answer to further clarify the severe dengue biological pathway.

To investigate the secondary aim of the study, logistic regression analysis was used to investigate the relationship of presence and absence of hemorrhage/vascular leakage with absolute monocyte count considering the

potential confounding effects of independent risk factors. The analysis included assessing the distance of study participant's residence from Hospital Ampang and the age variable as both categorical and continuous. Assumptions of the methodology were graphically assessed and fit of the model determined.

The logistic regression analysis did not produce an acceptable model to explain the observed relationship in the data and no significant or meaningful non-significant associations were established. While disappointing, the outcome is not entirely unexpected with the variation in the literature findings and complex nature of the dengue immune response. Future studies need to employ clear hypotheses that pick apart the biological pathway with well designed studies. After the a comprehensive severe dengue biological pathway has been hypothesized, a large study that has ample power to detect the association of interest maybe employed in a similar manner as the above analysis to determine the effect of each independent risk factor.

Limitations

The primary objective of the study was to assess the relationship of absolute monocyte count with primary and secondary dengue infection status. The study findings have restricted inference due to several limitations.

The literature review did not find another published article examining the study's hypothesis, potentially making this a novel investigation. As the study

was conducted in a single population in Malaysia, further research is necessary to assess applicability of the study findings to other populations within and outside of Malaysia.

Dengue infection is unpredictable and has a wide range of clinical manifestations, from asymptomatic to life threatening complications. The study targeted individuals aged 15 years and above who had symptoms severe enough to require hospitalization. Therefore, study findings can only be inferred to this population as it does not consider the population of individuals who develop mild or asymptomatic dengue disease or are aged less than 15 years.

The Malaysian healthcare system is two tiered: public and private. Conducting the study in a public hospital necessitates special consideration when determining to which populations the study findings can be inferred. Cost of care between public and private hospitals will undoubtedly dichotomize populations served. Also, differences in standard of care or medical capabilities in equipment or personnel may affect disease outcome.

There are opposing observations on whether or not SES and education are risk factors for dengue incidence, which suggests published results may be population dependent (Waterman *et al.,* 1985; Spiegel *et al.* 2007; Da Gloria Teixeira *et al.,* 2002). Furthermore, nutritional status, which is highly dependent on SES, has conflicting findings for the presence of decreased risk for severe dengue among malnourished populations (Kalayanarooj & Nimmannitya, 2005;

Maron *et al.,* 2010; Thisyakorn & Nimmannitya, 1993). Consequently, the inability to include SES and education in the study analysis, as the data was not available in hospital records from which study data was collected, may limit the inference of study findings to the general population.

Susceptibility for the development of severe dengue is largely dependent on the host immune response. The inclusion of the risk factors race, gender, and age in the study analysis will allow for consideration of biological differences between study participants. Inconsequentially, having not included education or SES will not have an effect on the internal validity of the study. Even with the discussed limitations, the study still has significant potential to contribute to knowledge of dengue disease.

Conclusions

The antibody-dependent enhancement and reactivation of memory T-cell hypotheses are currently the most comprehensive biological pathway models in the development of severe dengue manifestations. Under both hypotheses, a primary dengue infection modifies immunological components, which, during a secondary heterotypic infection, cause an excessive immune response resulting in disease complications. The dengue literature has provided evidence that monocytes are involved in the development of severe dengue; however, lacking

was analysis of whether monocyte involvement differed based on previous exposure to the dengue virus.

The study has observed a differential association of venous absolute monocyte count with primary and secondary dengue infection during defervescence. Overall, absolute monocyte count is significantly increased in secondary compared to primary dengue infections. The association is increasingly modified by male gender and a distance of less than 12.5 km of the participant residence from Hospital Ampang. Additionally, no significant association was observed between absolute monocyte count with absence and presence of hemorrhage/vascular leakage.

The study epidemiological evidence that the monocyte immune response is enhanced in secondary infection compared to primary infection provides further support of both the involvement of antibody-dependent enhancement and reactivation of memory T-cell hypotheses in the development of severe dengue. The inability to detect a relationship of absolute monocyte count with absence and presence of hemorrhage/vascular leakage is not entirely unexpected and speaks to the complex multifactorial immune response resultant of a dengue infection.

Currently, phase three clinical trials are ongoing for a dengue vaccine; however, even with a potential future licensed dengue vaccine, coverage of the entire human population at risk is years into the future, if ever. Dengue has

shown to be able to be passed through vertical transmission within the vectors, meaning dengue, even in a population with complete vaccine coverage, will survive and remain a threat to future unvaccinated generations.

Beyond the development of an easily administrable and safe tetravalent dengue vaccine, the over-riding challenge of the dengue research community is to understand the severe dengue biological pathway. A comprehensive understanding of the biological pathway will greatly aid researchers in developing medical interventions to lessen or prevent severe dengue complications after a dengue susceptible has been infected. Having a tetravalent dengue vaccine and specific medicines to reduce the risk of severe complications will provide public health professionals the necessary tools to effectively reduce the burden of dengue on the human population.

REFERENCES

Adams, B., Holmes, E. C., Zhang, C., Mammen, M. P., Nimmannitya, S., Kalayanarooj, S., & Boots, M. (2006). Cross-protective immunity can account for the alternating epidemic pattern of dengue virus serotypes circulating in Bangkok. *Proceeding of the National Academy of Sciences of the United States of America, 103,* 14234-14239. doi:10.1073/pnas.0602768103

Alirol, E., Getaz, L., Stoll, B., Chappuis, F, & Loutan, L. (2010). Urbanization and infectious diseases in a globalised world. *The Lancet Infectious Disease, 11,*131–141. doi:10.1016/S1473-3099(10)70223-1

Allen, A. (2007). *Vaccine: The controversial story of medicine's greatest lifesaver.* New York, NY: W.W. Norton.

Ang, K. T., Rohani, I., & Look, C.H. (2010). Role of primary care providers in dengue prevention and control in the community. *The Medical Journal of Malaysia, 65,* 58-62.

Appanna, R., Ponnampalavanar, S., Lum Chai See, L., & Sekaran, S. D. (2010). Susceptible and protective HLA class 1 alleles against dengue fever and dengue hemorrhagic fever patients in a Malaysian population. *PLoS ONE, 5,* e13029. doi:10.1371/journal.pone.0013029

Aremelagos, G. J. (1998). The viral superhighway. *The Sciences, 38,* 24-30. Retrieved from http://www.anthropology.emory.edu/FACULTY/ANTGA/Web%20Site/PDFs/Viral%20Superhighway.pdf

Arias, J. R. (2002). *Dengue how are we doing?: Celebrating 100 years of PAHO: Pan American Health Organization: 1902-2002* [PowerPoint slides]. Retrieved from www.paho.org/english/ad/dpc/cd/densit.ppt

Avirutnan, P., Punyadee, N., Noisakran, S., Komoltri, C., Thiemmeca, S., Auethavornanan, K., … Malasit, P. (2006). Vascular leakage in severe dengue virus infections: A potential role for the nonstructural viral protein NS1 and complement. *The Journal of Infectious Diseases, 193,* 1078-88. doi: 10.1086/500949

Ballenger-Browning, K. K., & Elder, J. P. (2009). Multi-modal Aedes aegypti mosquito reduction interventions and dengue fever prevention. *Tropical Medicine & International Health, 14,* 1542–1551. doi:10.1111/j.1365-3156.2009.02396.x

Barniol, J., Gaczkowski, R., Vega Barbato, E., da Cunha, R. V., Salgado, D., Martinez, E. … Jaenisch, T. (2011). Usefulness and applicability of the revised dengue case classification by disease: multi-centre study in 18 countries. *BMC Infectious Diseases, 11,* e106. doi: 10.1186/1471-2334-11-106

Barrett, R., Kuzawa, C. W., McDade, T., & Armelagos, G. J. (1998). Emerging and re-emerging diseases: The third epidemiologic transition. *Annual Review of Anthropology, 27*, 247-271. Retrieved from http://www.epa.gov/ncer/biodiversity/pubs/ann_vol27_247.pdf

Beaumier, C. M., Mathew, A., Bashyam, H. S., & Rothman, A. L. (2008). Cross-reactive memory $CD8^+$ T cells alter the immune response to heterologous secondary dengue virus infections in mice in a sequence-specific manner. *The Journal of Infectious Diseases, 197*(4), 608-617. doi:10.1086/526790

Böttcher, H. M. (1964). *Wonder drugs: A history of antibiotics.* Philadelphia, PA: Lippincott.

Burnet, F. M., & White, D. O. (1962). *Natural History of Infectious Disease.* Cambridge, UK: Cambridge University Press.

Central Intelligence Agency (CIA). (2011). Malaysia. Retrieved from https://www.cia.gov/library/publications/the-world-factbook/geos/my.html

Center for Disease Control and Prevention (CDC). (2010). *Clinical Guidance.* Retrieved from http://www.cdc.gov/dengue/clinicalLab/clinical.html

Center for Disease Control and Prevention (CDC). (2011). *Dengue.* Retrieved from http://www.cdc.gov/Dengue/

Chadee, D. D., Williams, F. L. R., & Kitron, U. D. (2005). Impact of vector control on a dengue fever outbreak in Trinidad, West Indies, in 1998. *Tropical Medical & International Health, 10,* 748–754. doi:10.1111/j.1365.2005.01449.x

Chin, J. (2000). *Control of communicable diseases manual (17th ed.).* Washington, D.C.: American Public Health Association.

Chua, G. (2010, September 1). Dengue victims usually older. *The Strait Times.* Retrieved from http://www.straitstimes.com/BreakingNews/Singapore/Story/STIStory_573660.html

Cockburn A. (1963). The Evolution and Eradication of Infectious Diseases [Abstract]. *The Bulletin of Hygiene, 39.* Retrieved from http://www.cabdirect.org/abstracts/19642902283.html

Cockburn, T. A. (1971). Infectious disease in ancient populations [Abstract]. *Current Anthropology, 12,* 45-62. Retrieved from http://www.jstor.org/stable/2740635?seq=1

Cohen, M. L. (2000). Changing patterns of infectious disease. *Nature, 406,* 762-767. doi:10.1038/35021206

Corbett, E. L., Watt, C. J., Walker, N., Maher, D., Williams, B. G., Raviglione, M. C., & Dye, C. (2003). The growing burden of tuberculosis - Global trends and interactions with the HIV epidemic. *Archives of Internal Medicine, 163,* 1009-1021. Retrieved from http://archinte.ama-assn.org.ezproxy.hsc.usf.edu/cgi/reprint/163/9/1009

Cummings, D. A. T., Iamsirithaworn, S., Lessler, J. T., McDermott, A., Prasanthong, R., Nisalak, A., … Gibbons, R. V. (2009). The impact of the demographic transition on dengue in Thailand: Insights from a statistical analysis and mathematical modeling. *PLoS Medicine, 6, e1000139.* doi:10.1371/journal.pmed.1000139

Davis, J. R., & Lederberg, J. (Eds.). (2000). Public health systems and emerging infections: Assessing the capabilities of the public and private sectors. Retrieved from http://www.nap.edu/openbook.php?record_id=9869

De Paula, S. O., & Fonseca, B. A. L. (2004). Dengue: A review of the laboratory tests a clinician must know to achieve a correct diagnosis. *The Brazilian Journal of Infectious Diseases, 8,* 390-398. doi:10.1590/S1413-86702004000600002

De Schweinitz, P. (2010). Is there a shaman in the house? *The Journal of the American Board of Family Medicine, 23,* 794-796. doi:10.3122/jabfm.2010.06.100064

Dengue Virus Net. (2011). *Antibody-dependent enhancement*. Retrieved from http://www.denguevirusnet.com/antibody-dependent-enhancement.html

Durbin, A. P., Vargas, M. J., Wanionek, K, Hammond, S. N., Gordon, A., Rocha, C, … Harris, E. (2008). Phenotyping of peripheral blood mononuclear cells during acute dengue illness demonstrates infection and increased activation of monocytes in severe cases compared to classic dengue fever. *Virology, 376,* 429–435. doi:10.1016/j.virol.2008.03.028

Dussart, P., Labeau, B., Lagathu, G., Louis, P., Nunes, M. R. T., Rodrigues, S. G., … Baril, L. (2006). Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. *Clinical and Vaccine Immunology, 13,* 1185-1189. doi:10.1128/CVI.00229-06

Encyclopedia Britannica, Inc. (2009). Population Density of Malaysia. In *Encyclopedia Britannica online*. Retrieved from http://www.britannica.com/EBchecked/topic-art/359754/128347/Population-density-of-Malaysia

Endy, T. P., Nisalak, A., Chunsuttiwat, S., Vaughn, D. W., Green, S., Ennis, F. A., … Libraty, D. H. (2004). Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. *The Journal of Infectious Diseases, 189,* 990–1000. doi:10.1086/382280

Epstein, P. (1995). Emerging diseases and ecosystem instability: New threats to public health. *American Journal of Public Health, 85,* 168-172. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1615306/

Farlex, Inc. (2011). *The free dictionary.* Retrieved from http://www.thefreedictionary.com/

Farrar, J., Focks, D., Gubler, D., Barrera, R., Guzman, M.G., Simmons, C., … Kalayanarooj, S. (2007). Editorial: Towards a global dengue research agenda. *Tropical Medicine and International Health, 12,* 695-699. Doi:10.1111/j.1365-3156.2007.01838.x

Fried, J. R., Gibbons, R.V., Kalayanarooj. S., Thomas, S. J., Srikiatkhachorn, A., Yoon, I … Cummings, D. A. T. (2010). Serotype-specific differences in the risk of dengue hemorrhagic fever: An analysis of data collected in Bangkok, Thailand from 1994 to 2006. *PLoS Neglected Tropical Diseases, 4,* e617. doi:10.1371/journal.pntd.0000617

Galea, S., & Tracy, M. (2007). Participation rates in epidemiologic studies. *Annals of Epidemiology, 17,* 643-653. doi:10.1016/j.annepidem.2007.03.013

Gamble, J., Bethell, D., Day, N. P., Loc, P. P., Phu, N. H., Gartside, I. B. … White, N. J. (2000). Age-related changes in microvascular permeability: A significant factor in the susceptibility of children to shock? *Clinical Science, 98,* 211–216. Retrieved from http://www.clinsci.org/cs/098/0211/0980211.pdf

George, R. (1987). Dengue haemorrhagic fever in Malaysia: A review [Abstract]. *The Southeast Asian Journal of Tropical Medicine and Public Health, 18,* 278-283.

George, R., & Lam, S. K. (1997). Dengue virus infection--the Malaysian experience [Abstract]. *Annals of the Academy of Medicine, Singapore, 26(6),* 815-819.

Green, S., Pichyangkul, S., Vaughn D. W., Kalayanarooj, S., Nimmannitya, S., Nisalak, A., … Ennis, F. A. (1999). Early CD69 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever. *The Journal of Infectious Diseases, 180,* 1429-1435. doi:10.1086/315072

Gubler, D. J. (1997). Dengue and dengue hemorrhagic. New York, NY: CAB International Press.

Gubler, D. J. (1998). Dengue and dengue haemorrhagic fever. *Clinical Microbiology Reviews, 11,* 480–496. Retrieved from http://cmr.asm.org/cgi/content/full/11/3/480

Gubler, D. J. (2004). The changing epidemiology of yellow fever and dengue, 1900 to 2003: full circle? *Comparative Immunology, Microbiology and Infectious Diseases, 27,* 319-330. doi:10.1016/j.cimid.2004.03.013

Guha-Sapir, D., & Schimmer, B. (2005). Dengue fever: New paradigms for a changing epidemiology. *Emerging Themes in Epidemiology, 2,* 1-10. doi:10.1186/1742-7622-2-1

Guy, B., Almond, J., & Lang, J. (2011). Dengue vaccine prospects: A step forward. The Lancet, 377, 381-382. doi:10.1016/S0140-6736(11)60128-1

Guzman, A.,& Istúriz, R. E. (2010). Update on the global spread of dengue. *International Journal of Antimicrobial Agents, 36S,* S40–S42. doi:10.1016/j.ijantimicag.2010.06.018

Guzman, M. G., Kouri, G., & Halstead, S. B. (2000). Do escape mutants explain rapid increases in dengue case-fatality rates within epidemics? *The Lancet, 355,* 1902–1903. doi:10.1016/S0140-6736(00)02303-5

Guzman, M. G., Kouri, G., Valdes, L., Bravo, J., Vazquez, S., & Halstead, S. B. (2002). Enhanced severity of secondary dengue-2 infections: Death rates in 1981 and 1997 Cuban outbreaks. *Pan American Journal of Public Health, 11,* 223-227. Retrieved from http://www.scielosp.org/pdf/rpsp/v11n4/10467.pdf

Hales, S., de Wet, N., Maindonald, J., & Woodward, A. (2002). Potential effect of population and climate changes on global distribution of dengue fever: An empirical model. *The Lancet, 360,* 830–834. *doi:10.1016/S0140-6736(02)09964-6*

Halstead, S. B. (1994). Dengue and dengue hemorrhagic fever. In G. W. Beran (Ed.), *Handbook of Zoones, Section B: Viral (2nd ed)* (89-100). Retrieved from http://books.google.com/books?hl=en&lr=&id=hDxeXDisYKEC&oi=fnd&pg=PA89&dq=Halstead,+1994+dengue&ots=cX2GaqtJHq&sig=2PY3Lh2mg2anl5N71fGBgb6OBWA#v=onepage&q=Halstead%2C%201994%20dengue&f=false

Halstead, S. B., Mahalingam, S., Marovich, M. A., Ubol, S., & Mosser, D. M. (2010). Intrinsic antibody-dependent enhancement of microbial infection in macrophages: Disease regulation by immune complexes. *The Lancet Infectious Diseases, 10*, 712-22. doi:10.1016/S1473-3099(10)70166-3.

Halstead, S. B., Streit, T. G., Lafontant, J. G., Putvatana, R., Russell, K., Sun, W., … Watts, D. M. (2001). Haiti: Absence of dengue hemorrhagic fever despite hyperendemic dengue virus transmission. *The American Journal of Tropical Medicine and Hygiene, 65,* 180–183. Retrieved from http://www.ajtmh.org/cgi/reprint/65/3/180

Hayward, A. C., & Coker, R. J. (2008). Could a tuberculosis epidemic occur in London as it did in New York? *Emerging Infectious Diseases, 6,* 12-16. Retrieved from http://www.cdc.gov/ncidod/eid/vol6no1/hayward.htm

Hempel, S. (2007). *The strange case of the Broad Street pump: John Snow and the mystery of cholera.* Berkeley, CA: University of California Press.

Hussin, N., Jaafar, J., Naing, N. N., Mat, H. A., Muhamad, A. H., & Mamat, M. N. (2005). A review of dengue fever incidence in Kota Bharu, Kelantan, Malaysia during the years 1998-2003. *The Southeast Asian Journal of Tropical Medicine and Public Health, 36,* 1179-1186. Retrieved from http://www.tm.mahidol.ac.th/seameo/2005_36_5/16-3538.pdf

Imhof B. A., & Aurrand-Lions, M. (2004). Adhesion mechanisms regulating the migration of monocytes. *Nature Reviews Immunology, 4,* 432-444. doi:10.1038/nri1375

Innis, B. L. (1997). Antibody response to virus infection. In Gubler, D. J. and Kuno, G. (Eds.), *Dengue and Dengue Hemorrhagic Fever*, New York, NY: CAB International.

Jansen, C. C., & Beebe, N. W. (2010). The dengue vector *Aedes aegypti*: What comes next. *Microbes and Infection, 12,* 272-279. doi:10.1016/j.micinf.2009.12.011

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature, 451,* 990-994. doi:10.1038/nature06536

Jouanna, J. (1999). *Hippocrates.* Baltimore, MD: Johns Hopkins University Press.

Kalayanarooj, S. & Nimmannitya, S. (2005). Is dengue severity related to nutritional status? *The Southeast Asian Journal of Tropical Medicine and Public Health, 36,* 378-384.

Kalayanarooj, S., Vaughn, D. W., Nimmannitya, S., Green, S., Suntayakorn, S., Kunentrasai, N., … & Ennis F. A. (1997). Early clinical and Laboratory indicators of acute dengue illness. *The Journal of Infectious Diseases, 176,* 313–321. doi: 10.1086/514047

Kaplan G., & Keil, J. (1993). Socioeconomic actors and cardiovascular disease: A review of the literature. *Circulation. 88,* 1973-1988. Retrieved at http://circ.ahajournals.org.ezproxy.hsc.usf.edu/cgi/reprint/88/4/1973

King, C. A., Anderson, R., & Marshall, J. S. (2002). Dengue virus selectively induces human mast cell chemokine production. *Journal of Virology, 76,* 8408-8419. doi: 10.1128/JVI.76.16.8408-8419.2002

Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S. … Fridkin, S. K. (2007). Invasive methicillin-resistant *staphylococcus aureus* infections in the United States. *The Journal of the American Medical Association, 298,* 1763-1771. doi: 10.1001/jama.298.15.1763

Knapp, V. J. (1989). *Disease and its impact on modern European history* (Vol. 10). Lewiston, NY: Mellen.

Kumar, A., Rao, C. R., Pandit, V., Shetty, S., Bammigatti, C., & Samarasinghe, C. M. (2010). Clinical manifestations and trend of dengue cases admitted in a tertiary care hospital, Udupi district, Kamataka. *Indian Journal of Community Medicine, 35,* 386-390. doi: 10.4103/0970-0218.69253

Kupperberg, P. (2008). *The influenza pandemic of 1918-1919*. New York, NY: Chelsea House Publishers.

Kyle, J. L., & Harris, E. (2008). Global spread and persistence of dengue. *Annual Review of Microbiology, 62,* 71-92. doi:10.1146/annurev.micro.62.081307.163005

Last, J. M. (1988). *A dictionary of epidemiology (2nd ed.).* Oxford, UK: Oxford University Press.

Lederberg, J. (1997). Infectious disease as an evolutionary paradigm. *Emerging Infectious Diseases, 3,* 417-423. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2640075/pdf/9366592.pdf

Leong, A. S., Wong, K. T., Leong, T. Y., Tan, P. H., & Wannakrairot, P. (2007). The pathology of dengue hemorrhagic fever [Abstract]. *Seminars in Diagnostic Pathology 24,* 227-236. doi:10.1053/j.semdp.2007.07.002

Libraty, D. H., Endy, T. P., Houng, H. S., Green, S., Kalayanarooj, S., Suntayakorn, S., … Rothman, A. L. (2002). Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. *The Journal of Infectious Disease 185,* 1213-1221. doi: 10.1086/340365

Link, K. (2005). *The vaccine controversy: The history, use, and safety of vaccinations*. Westport, CT: Praeger Publishers.

Liu, C., Huang, K, Huang, M, Lin, J. J., Wang, S. M., Liu, J., … & Lei, H. (2008). High case-fatality rate of adults with dengue hemorrhagic fever during an outbreak in non-endemic Taiwan: Risk factors for dengue-infected elders. *American Journal of Infectious Diseases, 4,* 10-17. Retrieved from http://www.scipub.org/fulltext/ajid/ajid4110-17.pdf

Lowy, F. D. (2010). Mapping the distribution of invasive staphylococcus aureus across Europe. *PLoS Medicine, 7,* e1000205. doi:10.1371/journal.pmed.1000205

Lum, L. C., Suaya, J. A., Tan, L. H., Sah, B. K., & Shepard, D. S. (2008). Quality of life of dengue patients. *The American Journal of Tropical Medicine and Hygiene, 78,* 862-867. Retrieved from http://www.ajtmh.org/cgi/reprint/78/6/862

Lupi, O. (2011). Mosquito-borne hemorrhagic fevers. *Clinics in Dermatology, 29,* 33–38. doi:10.1016/j.det.2010.09.009

Lye, D. C., Chan, M., Lee, V. J., & Leo, Y. S. (2008). Young adults with uncomplicated dengue fever need hospitalization? A retrospective analysis of clinical and laboratory features. *Singapore Medical Journal, 49,* 476-479.

Malaysian Ministry of Health (MMOH). (2011). Ministry of Health. Retrieved from http://www.moh.gov.my/

Mangill, A., & Gendreau, M. A. (2005). Transmission of infectious diseases during commercial air travel. *The Lancet, 365,* 989–996. doi:10.1016/S0140-6736(05)71089-8

Maron, G. M., Clara, A. W., Diddle, J. W., Pleites, E. B., Miller, L., Macdonald, G., & Adderson, E. E. (2010). Association between nutritional status and severity of dengue infection in children in El Salvador. *The American Journal of Tropical Medicine and Hygiene, 82,* 324-329.

Martina, B. E., Koraka, P., & Osterhaus, A. D. (2009). Dengue virus pathogenesis: An integrated view. *Clinical Microbiology Reviews, 22,* 564–81. doi:10.1128/CMR.00035-09

McNeill, W. H. (1976). *Plagues and People.* Garden City, NY: Anchor/Doubleday.

Metchnikoff, E. (1971). *The founders of modern medicine: Pasteur, Koch, Lister.* Freeport, NY: Books for Libraries Press.

Moellering, R. C. Jr. (1995). Past, present, and future of antimicrobial agents. *The American Journal of Medicine, 29,* 11S-18S.

Monath, T. P. (2007). Dengue and yellow fever - Challenges for the development and use of vaccines. *New England Journal of Medicine, 357,* 2222-2225. Retrieved from http://www.nejm.org/doi/pdf/10.1056/NEJMp0707161

Mongkolsapaya, J., Duangchinda, T., Dejnirattisai, W., Vasanawathana, S., Avirutnan, P., Jairungsri, A. … Screaton, G. (2006). T cell responses in dengue hemorrhagic fever: Are cross-reactive T cells suboptimal? *Journal of Immunology, 176,* 3821–3829. Retrieved from http://www.jimmunol.org.ezproxy.hsc.usf.edu/content/176/6/3821.full.pdf+html

Moore, K., & Tabas, I. (2011). Macrophages in the pathogenesis of atherosclerosis. *Cell. 145,* 341-355. doi:10.1016/j.cell.2011.04.005

Nagao, Y., & Koelle, K. (2008). Decreases in dengue transmission may act to increase the incidence of dengue hemorrhagic fever. *Proceedings of the National Academy of Sciences of the United States of America, 105,* 2238–2243. doi: 10.1073/pnas.0709029105

National Institute of Allergy and Infectious Diseases (NIAID). (2010). *Emerging and re-emerging infectious diseases: List of NIAID emerging and re-emerging diseases group I—pathogens newly recognized in the past two decades.* Retrieved form http://www.niaid.nih.gov/topics/emerging/Pages/list.aspx

National Institutes of Health (NIH). (2011). Regulations and ethical guidelines: The Belmont report: Ethical principles and guidelines for the protection of human subjects of research. Retrieved from http://ohsr.od.nih.gov/guidelines/belmont.html

Newland, K. (1981). *Infant mortality and the health of societies.* Washington, D.C: Worldwatch Institute. Retrieved from http://www.eric.ed.gov/PDFS/ED210114.pdf

Nielsen, D, G. (2009). The relationship of interacting immunological components in dengue pathogenesis. Virology Journal, *6*, 211. doi:10.1186/1743-422X-6-211.

Nunn, J. F. (2002). *Ancient Egyptian medicine.* Norman, OK: University of Oklahoma Press.

Oleckno, W. A. (2002). *Essential epidemiology: Principles and applications.* Long Grove, IL: Waveland Pres, Inc.

Ooi, E., Goh, K., & Gubler, D. J. (2006). Dengue prevention and 35 years of vector control in Singapore: Dengue in adults. *Emerging Infectious Diseases, 12,* 887-893. Retrieved from http://www.cdc.gov/ncidod/EID/vol12no06/05-1210.htm

Omran, A. R. (1971). The epidemiologic transition: A theory of the epidemiology of population change. *The Milbank Memorial Fund Quarterly, 49,* 509-538. Retrieved from http://www.milbank.org/quarterly/830418omran.pdf

Orthman, N. (2007). Clinical profile of dengue infection in children versus adults [Abstract]. *Paediatric viral infections,* S435. Retrieved from http://www.blackwellpublishing.com/eccmid17/abstract.asp?id=57346

PanBioDengue. (2011). *History of dengue.* Retrieved from www.panbiodengue.com

Paupy, C., Delatte, H., Bagny, L., Corbel, V., & Fontenille, D. (2009). Aedes albopictus, an arbovirus vector: From the darkness to the light. *Microbes and Infection, 11,* 1177-1185. doi:10.1016/j.micinf.2009.05.005

Pang, T., Cardosa, M. J. & Guzman, M. G. (2007). Of cascades and perfect storms: The immunopathogenesis of dengue haemorrhagic fever-dengue shock syndrome (DHF/DSS). *Immunology and Cell Biology, 85,* 43–45. doi:10.1038/sj.icb.7100008

Peeling, R. W., Artsob, H., Pelegrino, J. L., Buchy, P., Cardosa, M. J., Devi, S.… Yoksan, S. (2010). Evaluation of diagnostic tests: Dengue. *Nature Reviews Microbiology,* S30-S38. doi:10.1038/nrmicro2459

Pengsaa, K., Yoksan, S., Limkittikul, K., Wisetsing, P., Sirivichayakul, C., Hutacharoen, P. … Sabchareon, A. (2003). Maternally transferred neutralising dengue antibodies in Thai infants: A pilot study. *Annals of Tropical Pediatrics, 23,* 159-165. doi:10.1179/027249303322296466

Pennsylvania State University (PSU). (2010). *Epidemiological Triad.* Retrieved from https://onlinecourses.science.psu.edu/stat507/node/25

Perera, R., & Kuhn, R. J. (2008). Structural proteomics of dengue virus. *Current Opinion in Microbiology, 11,* 369–377. doi:10.1016/j.mib.2008.06.004

Pinheiro, F. P., & Corber, S. J. (1997). Global situation of dengue and dengue haemorrhagic fever, and its emergence in the Americas [Abstract]. *World Health Statistics Quarterly Report, 50*, 161-169. Retrieved from http://www.ncbi.nlm.nih.gov.ezproxy.hsc.usf.edu/pubmed?term=Pinheiro%20%26%20corber%2C%201997%20dengue

Polgar, S. (1964). Evolution and the ills of mankind. *Horizons of Anthropology*, (Ed.) Sol Tax. Chicago, IL: Aldine.

Poovaneswari, S. (1993). Dengue situation in Malaysia. *Malaysian Journal of Pathology, 15*(1), *3-7.* Retrieved from http://www.mjpath.org.my/past_issue/MJP1993.1/dengue%20situation%20in%20malaysia.pdf

Potts, J. A., Gibbons, R. V., Rothman, A. L., Srikiatkhachorn, A., Thomas, S. J., Supradish, P. … Kalayanarooj, S. (2010). Prediction of Dengue Disease Severity among Pediatric Thai Patients Using Early Clinical Laboratory Indicators. *PLoS Neglected Tropical Diseases, 4, e769.* doi:10.1371/journal.pntd.0000769

PubMed Health. (2009). *Severe acute respiratory syndrome (SARS).* Retrieved from http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0004460/

Qi, R. F., Zhang, L., & Chi, C. W. (2008). Biological characteristics of dengue virus and potential targets for drug design. *Acta biochimica et biophysica Sinca, 40*, 91-101. doi: 10.1111/j.1745-7270.2008.00382.x

Reves, R. (1985). Declining fertility in England and Wales as a major cause of the 20th-century decline in mortality - the role of changing family-size and age structure in infectious-disease mortality in infancy [Abstract]. *American Journal of Epidemiology, 122,* 112–126. Retrieved from http://aje.oxfordjournals.org/content/122/1/112.abstract

Rigau-Perez, J. (2006). Severe dengue: the need for new case definitions. *The Lancet Infectious Diseases*, 6, 297–302. doi:10.1016/S1473-3099(06)70465-0

Rigau-Perez, J. G., Clark, G. C., Gubler, D. J., Reiter, P., Sanders, E. J., & Vorndam, A. V. (1998). Dengue and dengue haemorrhagic fever. *The Lancet, 352*(9132), 971-977. doi:10.1016/S0140-6736(97)12483-7.

Rodenhuis-Zybert, I. A., Wilschut, J. & Smit, J. M. (2010). Dengue virus life cycle: Viral and host factors modulating infectivity. *Cellular and Molecular Life Sciences, 67,* 2773-2786. doi:10.1007/s00018-010-0357-z

Rothman, A. L. (2010). Cellular immunology of sequential dengue virus infection and its role in disease pathogenesis [Abstract]. *Current Topics in Microbiology and Immunology, 338,* 83-98. doi: 10.1007/978-3-642-02215-9_7

Rothman, A. L., & Ennis, F. A. (1999). Immunopathogenesis of dengue hemorrhagic fever. *Virology, 257,* 1–6. doi:10.1006/viro.1999.9656

Rudnick, A., & Lim, T. W. (1986). Dengue fever studies in Malaysia. *Institute for Medical Research Malaysia Bulletin, 23,* 51–152. Retrieved from http://infosvr.moh.gov.my:8070/vital/access/manager/Repository/EJ:6?exact=creator%3a%22T.W.+%28ed%29%2c+Lim%22

Russell, R.C., Webb, C.E., Williams, C. R., & Richie, S.A. (2005). Mark-release-recapture study to measure dispersal of the mosquito *Aedes aegypti* in Cairns, Queensland, Australia*. Medical and Veterinary Entomology, 19,* 451-457. doi:10.1111/j.1365-2915.2005.00589.x

Sabin, A. B. (1952). Research on dengue during World War II [Abstract]. *The American Journal of Tropical Medicine and Hygiene, 1,* 30–50. Retrieved from http://www.ajtmh.org/cgi/content/abstract/1/1/30

Salazar M. I., Richardson, J. H., Sánchez-Vargas, I., Olson, K. E. & Beaty, B. J. (2007). Dengue virus type 2: Replication and tropisms in orally infected *Aedes aegypti* mosquitoes. *BMC Microbiology, 7,* 9. doi:10.1186/1471-2180-7-9

Saw, S. H. (2007). The Population of Malaysia. Retrieved from http://books.google.com/books?hl=en&lr=&id=e4Yp2QJNVWgC&oi=fnd&pg=PR5&dq=The+Population+of+Malaysia,+Saw+Swee-Hock&ots=FbmW0LkABq&sig=LxtHt2oKWenBn7W4CVGd0gYjyfg#v=onepage&q&f=false

Shilts, R. (1988). *And the band played on: Politics, people, and the AIDS epidemic*. New York, N.Y.: Penguin Books.

Sierra, B., Kouri, G., & Guzman, M. G. (2007). Race: A risk factor for dengue hemorrhagic fever. *Archives of Virology, 152,* 533–542. doi:10.1007/s00705-006-0869-x

Skae, F. M. T. (1902). Dengue fever in Penang. *British Medical Journal, 2,* 1581-1582. Retrieved from http://www.ncbi.nlm.nih.gov.ezproxy.hsc.usf.edu/pmc/articles/PMC2402112/pdf/brmedj08482-0009b.pdf

Smith, C. E. (1956). The history of dengue in tropical Asia and its probable relationship to the mosquito Aedes aegypti. *The Journal of Hygiene, 59, 243-251.*

Snowden, F. M. (2008). Emerging and reemerging diseases: A historical perspective. *Immunological Reviews, 225,* 9-26. doi:10.1111/j.1600-065X.2008.00677.x

Spellberg, B. (2008). Dr. William H. Stewart: Mistaken or maligned? *Clinical Infectious Diseases, 47,* 294. Retrieved from http://cid.oxfordjournals.org.ezproxy.hsc.usf.edu/content/47/2/294.1.full.pdf+html

Spiegel, J. M., Bonet, M., Ibarra, A., Paglicca, N., Ouellette, V., & Yassi, A. (2007). Social and environmental determinants of Aedes aegypti infestation in Central Havana: results of a case–control study nested in an integrated dengue surveillance programme in Cuba. *Tropical Medicine and International Health, 12,* 503-510. doi:10.1111/j.1365-3156.2007.01818.x

Statistical Solutions. (2011). *Power and sample size calculator.* Retrieved from http://www.statistical solutions.net/pss_calc.php

Suaya, J. A., Shepard, D. S., & Beatty, M. A. (2006). *Dengue: Burden of disease and costs of illness.* Scientific working group report on dengue: Meeting report, 1-5 October 2006, Geneva, Switzerland. Retrieved from http://apps.who.int/tdr/svc/publications/tdr-research-publications/swg-report-dengue

Teo, D. & Ng, L. C. & Lam, S. (2009). Is dengue a threat to the blood supply? *Transfusion Medicine, 19,* 66–77. doi: 10.1111/j.1365-3148.2009.00916.x

Tee, H. P., How, S. H., Jamalludin, A. R., Safhan, M. N., Sapian, M. M., Kuan, Y. C., & Sapari, S. (2009). Risk factors associated with development of dengue haemorrhagic fever or dengue shock syndrome in adults in Hospital Tengku Ampuan Afzan Kuantan. *Medical Journal of Malaysia, 64(4),* 316-320. Retrieved from http://www.e-mjm.org/2009/v64n4/Dengue_Haemorrhagic_Fever.pdf

Thayan, R., Huat, T. L., See, L. L., Tan, C. P., Khairullah, N. S., Yusof, R., & Devi, S. (2009). The use of two-dimension electrophoresis to identify serum biomarkers from patients with dengue haemorrhagic fever. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 103,* 413-419. doi:10.1016/j.trstmh.2008.12.018

The University of Texas Medical Branch (UTMB). (2009). Flaviviridae Family. Retrieved from http://www.utmb.edu/discoveringdenguedrugs-together/Flaviviridae%20Family.htm

The World Bank. (2011). *World development indicators.* Retrieved from http://data.worldbank.org/data-catalog/world-development-indicators?cid=GPD_WDI

Thenmozhi, V., Hiriyan, J. G., Tewari, S. C., Samuel, P. P., Paramasivan, R., Rajendran, R. … Tyagi, B. K. (2007). Natural vertical transmission of dengue virus in Aedes albopictus (Diptera: Culicidae) in Kerala, a southern Indian state. *Japanese Journal of Infectious Diseases, 60,* 245-249. Retrieved from http://www.nih.go.jp/JJID/60/245.pdf

Thisyakorn, U., & Nimmannitya, S. (1993). Nutritional status of children with dengue hemorrhagic fever. *Clinical Infectious Diseases, 16,* 295-297.

Thomas, L., Brouste, Y., Najioullah, F., Hochedez, P., Hatchuel, Y., Moravie, V., … Cabie, A. (2010). Predictors of severe manifestations in a cohort of adult dengue patients. *Journal of Clinical Virology, 48,* 96-99. doi:10.1016/j.jcv.2010.03.008

Tsai J. J., Chan K. S., Chang J. S., Chang K., Lin C. C., Huang J. H., … Lin C. Y. (2009). Effect of serotypes on clinical manifestations of dengue fever in adults. *Journal of Microbiology, Immunology and Infection, 42,* 471-478. Retrieved from http://www.ejmii.com/article_pdf.php?code=PDT4be0ff4e4cc8b

Ubol, S., & Halstead, S. B. (2010). How innate immune mechanisms contribute to antibody-enhanced viral infections. *Clinical and vaccine immunology, 17,* 1829–1835. Retrieved from http://www.jimmunol.org.ezproxy.hsc.usf.edu/content/176/6/3821.full.pdf+html

United Nations Educational, Scientific and Cultural Organization (UNESCO), (1985). World Congress on Youth. Retrieved from http://unesdoc.unesco.org/images/0006/000647/064796EB.pdf

United Nations Educational, Scientific and Cultural Organization (UNESCO). (1985). Activities of the United Nations system in the field of youth. *World Congress on Youth*. Retrieved from http://unesdoc.unesco.org/images/0006/000647/064796EB.pdf

UNICEF. (2011). Malaysia: Statistics. Retrieved from http://www.unicef.org/infobycountry/malaysia_statistics.html#75

United Nation (UN). (1990). Article 1, United Nation convention on the right of child. Retrieved from http://www2.ohchr.org/english/law/crc.htm

United Nations (UN). (2009a). UN: World population prospects: The 2009 revision and world urbanization prospects. Retrieved from http://esa.un.org/unpd/wup/index.htm

United Nations (UN). (2009b). World population prospects: The 2008 revision population database. Retrieved from http://esa.un.org/unpp

United Nations (UN). (2010, March). World urbanization prospects: The 2009 revision. Geneva, Switzerland: World Health Organization. Retrieved from http://esa.un.org/unpd/wup/index.htm

US Census Bureau. (2011). International data base: World population summary. Retrieved from http://www.census.gov/ipc/www/idb/worldpopinfo.php

Vasilakis, N. & Weaver, S. C. (2008). Chapter 1: The history and evolution of human dengue emergence. *Advances in Virus Research, 72,* 1-76. doi: 10.1016/S0065-3527(08)00401-6

Vaughn, D. W., Green, S., Kalayanarooj, S., Innis, B. L., Nimmannitya, S., Suntayakorn, S., … Nisalak, A. (2000). Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *Journal of Infectious Disease, 181,* 2-9. doi: 10.1086/315215

Vinomarlini, G., Rogayah, T., Saraswathy, T. S., Thayan, R., Apandi, M., Fauziah, M. K., & Saat, Z. (2011). Molecular typing of dengue viruses circulating on the east coast of peninsular Malaysia from 2005 to 2009 [Abstract]. *The Southeast Asian Journal of Tropical Medicine and Public Health, 42,* 94-99. Retrieved from http://www.tm.mahidol.ac.th/seameo/2011-42-1/13-4865.pdf

Vornham, V. & Kuno, G. (1997). Laboratory diagnosis of dengue virus infections. In Gubler, D. J. and Kuno, G., *Dengue and Dengue Hemorrhagic Fever*, New York, NY: CAB International.

Wade, R. (2010, October 31). Europe's plagues came from China, study finds. *The New York Times.* Retrieved from http://www.nytimes.com/2010/11/01/health/01plague.html?adxnnl=1&adxnnlx=1300122069-zc6FBSRuUaj96CxXSSmHwQ

Wang, E., Ni, H., Xu, R., Barrett, A. D., Watowich, S. J., Gubler, D. J., & Weaver, S. C. (2000). Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses. *Journal of Virology, 74,* 3227-3234. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC111823/pdf/jv003227.pdf

Waterman, S. H., Novak, R. J., Sather, G. E., Bailey, R. E., Rios, I., & Gubler, D. J. (1985). Dengue transmission in two Puerto Rico communities in 1982. *The American Journal of Tropical Medicine and Hygiene, 34,* 625-632.

Whitehorn, J. & Farrar, J. (2010). Dengue. *British Medical Bulletin, 95,* 161-173. doi: 10.1093/bmb/ldq019

Wichmann, O., Hongsiriwon, S., Bowonwatanuwong, C., Chotivanich, K., Sukthana, Y., & Pukrittayamee, S. (2004). Risk factors and clinical features associated with severe dengue infection in adults and children during the 2001 epidemic in Chonburi, Thailand. *Tropical Medicine & International Health, 9,* 1022–1029. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1365-3156.2004.01295.x/pdf

Wong, C. W., Wah Heng, C. L., Wan Yee, L., Soh, S. W. L., Kartasasmita, C. B., Simoes, E. A. F., … Miller, L. D. (2007). Optimization and clinical validation of a pathogen detection microarray. *Genome Biology, 8,* R93. doi:10.1186/gb-2007-8-5-r93

WordNet. (2011). *WordNet: A lexical database for English.* Retrieved from http://wordnet.princeton.edu/

World Health Organization (WHO). (1969). *The international health regulations.* Retrieved from http://www.who.int/ihr/current/en/index.html

World Health Organization (WHO). (1996). *The rise and spread of dengue, dengue hemorrhagic fever and its vectors, 1950-1990.* Retrieved from http://www.searo.who.int/en/Section10/Section332/Section519_2401.htm

World Health Organization (WHO). (1997). *Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd edition.* Retrieved from http://www.who.int/csr/resources/publications/dengue/Denguepublication/en/index.html

World Health Organization (WHO). (2005). *Preventing chronic diseases: A vital investment: WHO global report.* Retrieved from http://www.who.int/chp/chronic_disease_report/contents/en/index.html

World Health Organization (WHO). (2007). *The world health report 2007: A safer future: Global public health security in the 21st century.* Retrieved form http://www.who.int/whr/2007/whr07_en.pdf

World Health Organization (WHO). (2008). *The World Health Report 2008: Primary health care: Now more than ever.* Retrieved from http://www.who.int/whr/2008/en/index.html

World Health Organization (WHO) (2010). *Dengue, countries or areas at risk, 2010.* Retrieved from http://gamapserver.who.int/mapLibrary/Files/Maps/Global_DengueTransmission_ITHRiskMap.png

World Health Organization/Southeast Asia Region (WHO/SEAR). (2011). *Dengue/DHF: Case Fatality Rate(%) of DF/DHF in the South-East Asia Region (1985-2006).* Retrieved from http://www.searo.who.int/en/Section10/Section332_1102.htm

World Health Organization / Special Programme for Research and Training in Tropical Diseases (WHO/TDR). (2004). *Dengue Diagnostics: proceedings of a joint TDR/WHO and PDVI workshop.* Retrieved from http://apps.who.int/tdr/publications/tdr-research-publications/dengue-diagnostics-proceedings/pdf/dengue_diagnostics.pdf

World Health Organization / Special Programme for Research and Training in Tropical Diseases (WHO/TDR). (2007). Scientific working group report on Dengue, 1–5 October 2006, Geneva, Switzerland. Retrieved from http://www.who.int/tdr/publications/publications/swg_dengue_2.htm

World Health Organization/ Special Programme for Research and Training in Tropical Diseases (WHO/TDR). (2009). *Dengue: Guidelines for diagnosis, treatment, prevention and control.* Retrieved from http://whqlibdoc.who.int/publications/2009/9789241547871_eng.pdf

World Health Organization / Western Pacific Region (WHO/WPR). (2011). *Dengue in the Western Pacific. Manila, Philippines: World Health Organization.* Retrieved from http://www.wpro.who.int/health_topics/dengue/

World Tourism Organization (WTO). (2011). International Tourism 2010: Multi-speed recovery. Retrieved from http://85.62.13.114/media/news/en/press_det.php?id=7331&idioma=E

World Weather Information Service (WWIS). (2011). *Malaysia.* Retrieved from http://worldweather.wmo.int/020/c00082.htm

Yew, Y. W., Ye, T., Ang, L. W., Ng, L. C., Yap, G., James, L., … Goh, K. T. (2009). Seroepidemiology of dengue virus infection among adults in Singapore. *Annals Academy of Medicine Singapore, 38,* 667-675. Retrieved from http://www.annals.edu.sg/pdf/38VolNo8Aug2009/V38N8p667.pdf

Zakharov, S. V., & Ivanova, E. I. (2010). *Fertility decline and recent changes in Russia: on the threshold of the second demographic change.* Retrieved from http://www.rand.org/pubs/conf_proceedings/CF124/CF124.chap2.html#fn0

Ziegler, P. (1969). *The Black Death.* New York, NY: John Day Co.

APPENDICES

Appendix A: Epidemiological Transitions: The Human and Disease Relationship

An understanding of epidemiological transitions during human history will provide a foundation to the reader upon which to grasp and interpret the empirical evidence discerned in this study. The intended objective of the following section is to present a historic overview of the relationship between humans and disease as a backdrop for further, more in depth analysis of dengue epidemiology. The framework for the following discussion will use a theory expansion presented by Barrett and colleagues of Abdel Omran's originally presented theory of "epidemiological transition" or "health transition" (Omran, 1971; Barrett, Kuzawa, McDade, & Armelagos, 1998).

The epidemiology transition theory concentrates on major human morbidity and mortality patterns along with the "demographic, economic and sociologic determinants and consequences" (Omran, 1971). Barrett and colleagues (1998) identify three major epidemiological transitions during the period of time from our common hominoid ancestors until today, which incorporate many of Omran's original findings. To describe the period of time before the first epidemiological transition, Omran (1971) coined the phrase: "The

Age of Pestilence and Famine." This period of time began with the first hominoid ancestors on the African savanna lasting until the late-17th century. Characterizing "The Age of Pestilence and Famine" was a high mortality and birth rate. Population patterns were defined by epidemics and famine while remaining low in total population, as compared with the 20th & 21st centuries (Figure 1A, Phase 1).

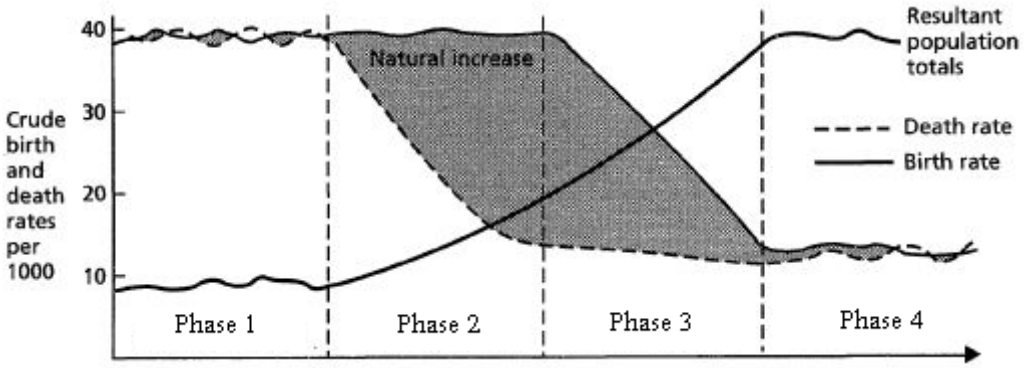


Figure 1A. Epidemiological transitions (UN, 2009a) edited.

"The Age of Pestilence and Famine" can be analyzed by conceptualizing a dichotomous break along food collection methods: foraging and agriculture. From the interpretations of archeologists and anthropologists, modern human pre-historic ancestors on the African savanna are thought to have lived in small nomadic tribes who foraged for food (Burnet & White, 1962). Population dynamics would have not favored disease organisms of low pathogenicity or high virulence as the organism would have not encountered a large enough

population to sustain transmission. Diseases which were likely to affect the early hominoids were enteric bacterium, transferred by the oral-fecal route, and head and body lice (Cockburn, 1971, as cited in Barrett *et al.,* 1998). As early hominids increased tool usage and dispersed throughout Europe, Asia and eventually colonizing much of the globe, they would have disturbed different ecological niches increasing their risk for contracting novel diseases. Sustained transmission of an emerging diseases resulting in an endemic nature in the hominoid population would have remained low during this period of foraging as populations would have remained below sustainable disease thresholds of transmission (Barrett *et al.*, 1998).

Approximately 10,000 years ago, the early nomadic tribes began to establish permanent settlements and no longer used foraging as a primary method for food collection, turning to agriculture (Barrett *et al.,* 1998). The transition to food cultivation had no significant effect on the high mortality and birth rates, however; they allowed for the development of slightly larger populations which introduced a number of new disease threats. An enlarged, sessile population allowed for increased contact between individuals and accumulation of waste (Barrett *et al.,* 1998). Furthermore, increased population densities would have allowed viral infections such as measles, smallpox and respiratory infections, along with vector-borne diseases (e.g. plague and typhus) to thrive. Cholera epidemics would have occurred with accumulated waste (Knapp, 1989). During this period, zoonotic diseases (e.g. tuberculosis, anthrax

and Q fever) would have emerged due to increased contact with domesticated animals (Polgar, 1964, as cited in Barrett *et al.,* 1998)

The first accounts on the origin of disease come from documents written by the great ancient civilizations of modern day Egypt, India, and China. Disease was generally thought of an external affliction caused either by the wrath of a well intentioned god(s) who the individual had upset, or the hex from an evil demon. This supernatural stance on the origin of disease was in turn treated with highly superstitious remedies, many of which did more harm than good to the afflicted (Nunn, 2002). Supernatural disease origin has not faded with antiquity but remains in present times, most observably in the practices of shamanism (de Schweinitz, 2010). Not until approximately 400 Before Common Era (BCE) in Greece, did a shift in philosophy towards a natural or internal origin of disease occur. Hippocrates, known as the "Father of Modern Medicine," has been given credit for this shift in disease philosophy, among many other significant contributions, forming a foundation for medical advancement (Jouanna, 1999).

By the 14th century of the Common Era (CE), humans had formed regional populations, interacting through trading routes over land and on the high seas (McNeill, 1976). While trade spurred economies, within the trading vessels tradesmen and vector alike carried diseases to new populations. Originating in China, the plague or Black Death travelled across the Asian continent and then throughout Europe (Wade, 2010). Plague, caused by the bacterium *Yersinia pestis*, is transmitted to humans through the bite of a flea, direct contact or

indirect droplet inhalation. The Black Death peaked during 1348-1350CE and is estimated to have killed 1/3 of the European population (Ziegler, 1969). The increasingly crowded and unsanitary conditions in combination with poor nutritional diets brought destructive epidemics throughout the remainder of the "Age of Pestilence and Famine" (McNeill, 1976).

The first major historical epidemiological transition began in the late seventeenth century in Europe which entered "The Age of Receding Pandemics" (Omran, 1971). As the name suggests, pandemics, while not absent, become less devastating as dietary, sanitation and hygiene efforts increase. Defining the era is a drastic drop in mortality rate and increase in life-expectancy which, when combined with a very high birth rate, resulted in a substantial natural increase in the total population (Figure 1A, Phase 2) (Omran, 1971). While increases in basic standards of living aided in the decreased mortality rate, without the intellectual contributions of John Snow, Louis Pateur and Robert Koch, improvements in human health may not have been sustained.

John Snow, an English physician, is considered to be one of the fathers of epidemiology for his work on a cholera outbreak in Soho, England (Hempel, 2007). A skeptic of the miasma theory, which stated that diseases where caused by "bad air," Snow used mapping of cholera cases and statistical analysis to show an association with the outbreak point source, a water pump (Hempel, 2007). Refuting "bad air," the disease origin dogma of the day, John Snow provided bases for further disease origin investigations.

Not until Louis Pastuer's experiment to refute spontaneous generation and subsequent statement of the Germ Theory of Disease during the 1860's, did the true causal relationship between disease and humans begin to become evident (Metchnikoff, 1971). The Germ Theory is based on the idea that disease is caused by the invasion of the body by microorganisms. In 1880, Robert Koch empirically validated Louis Pastuer's Germ Theory and proposed his own postulates on the matter of disease origin (Metchnikoff, 1971). Still used today, Koch's Postulates are a set of four criteria used to empirically determine if a microorganism is the causal agent for a disease.

Late into the 19th century and beginning of the 20th century, medical science was rapidly advancing the understanding of disease and beginning to produce effective primary prevention in the form of vaccines. Edward Jenner is given credit for having created the first vaccine against the smallpox virus through the method of variolation of cowpox scabs in 1796 Current Era (CE) (Allen, 2007). After a gap of almost a century, vaccine development progressed to address rabies (1884), cholera and typhoid (1896), plague (1897), and pertussis (1915) (Link, 2005). During this time period, medical science was beginning to feel they could solve infectious diseases; however, with the world at war the influenza pandemic of 1918 took the world and field of science by surprise. Unlike most infectious diseases which target the very young, elderly and immunocompromised, pandemic influenza of 1918 targeted healthy young adults (Kupperburg, 2008). Researchers of the day rushed to develop a vaccine

for influenza. Unfortunately, these vaccine attempts would prove unsuccessful dampened by the initial hypothesis that the pandemic was caused by a bacterium (Kupperburg, 2008).

Although the influenza pandemic of 1918 had exposed the medical and scientific communities for their limited prevention capabilities, following World War II North America was soon undergoing a second epidemiological transition. The transition was as a result in a surge of scientific and public health triumphs, including: the eradication of polio, improved sanitation requirements, federal oversight to further protect consumables in America (e.g. Federal Food, Drug, and Cosmetic Act, 1938, USA) and the continued discovery of new more powerful antibiotics (Bӧttcher, 1964).

”The Age of Degenerative and Man-Made Diseases”, is the second epidemiologic transition defined by a constant low mortality rate and decreasing birth rate (Omran, 1971). The decreasing birth and infant mortality rates are generally a result of increased access to healthcare and education for women and children (Newland, 1981). During this third transition period, the total population expands rapidly in the beginning, but as the birthrate drops, the population growth rate slows (Figure 1A, Phase 3). Sustained public health efforts see a continued drop in infectious disease mortality, combined with the increased lifespan, results in chronic diseases, for the first time, becoming the leading cause of mortality (Omran, 1971). Chronic diseases with high prevalence in this stage include cancer, diabetes, coronary artery disease and

chronic obstructive pulmonary disorder (Kaplan & Keil, 1993). These diseases have a multitude of risk factors, including tobacco, alcohol, human made environment pollution (i.e. air, water & noise), diet and decreased exercise.

The transition between the receding pandemic and man-made disease eras is not typically as abrupt as written in this thesis. Low-income countries may not have been able to afford to invest enough in their public health infrastructure to rid their country of infectious disease epidemics. At the same time, globalization (e.g. increased pollution, availability of tobacco and alcohol) and the westernization of the diet (i.e. increased sugar, salt and fat [especially from animals] intake) increase the burden of chronic diseases on the population. This double burden is evident by the WHO reporting that 80% of chronic disease deaths occur in low and middle income nations (WHO, 2005). There is a continued need to address both chronic and infectious disease problems simultaneously.

Snowden (2008) has deemed the period since the discovery of antibiotics in the 1940's: "The Age of Hubris." While the tenth Surgeon General of the United States has been incorrectly quoted as saying it was "time to close the books on infectious diseases" (Spellberg, 2008), this attitude was in fact the overwhelming mood of world leaders of the day demonstrated by the US Secretary of State George Marshall declaring in 1948 that the world had the ability to rid itself of infectious diseases (Lederberg, 1997; Snowden, 2008). Also during this period, Aidan Cockburn, an epidemiologist at John Hopkins University

and WHO advisor, even went as far as to give a timetable for complete infectious disease eradication: achieved by “2060” (Cockburn, 1963, as cited in Barrett *et al.,* 1998).

The feeling of inevitable conquest over infectious diseases was being fueled by the post-World War II social uplift in earnings, education, diet and sanitation, food production, the discovery of the “magic bullet” for malaria - quinine, WHO announcing in 1979 the eradication of smallpox, and the discovery of penicillin and streptomycin. Beyond public euphoria over accomplishments, the scientific community’s incorrect view of the stagnant or slow-paced evolutionary process towards benign microbes further eroded the perceived threat of infectious diseases (Snowden, 2008). Feelings translated into action as the 1969 International Health Regulations only required three reportable diseases: yellow fever, plague and cholera (WHO, 1969).

As global conditions changed, researchers have expanded Omran’s original model to fit and explain developments. One such expanded model, described by Zakharov and Ivanova (2010), attempts to explain changes to Russian epidemiology. Russia, having undergone a social upheaval, has seen a drop in its population as a result of stagnate to decreasing birth rate with an increased death rate. Seen in other developed nations with social upheaval, the increased death rate is not only due to chronic diseases, but resurgence in infectious diseases as a result of a failure in governmental public health programs. These factors have lead to an overall decrease in the total population.

The above model, while imperative to understanding overall global epidemiological transitions, does not explain the emerging and re-emerging pattern in infectious diseases observed within the last half century on a global level.

In post-World War II, individuals who held the belief that infectious diseases would drive themselves by means of natural selection towards a benign existence were termed “eradicationists” (Snowden, 2008). The decrease virulence of syphilis from the 16th century compared to the mid-twentieth century was a pillar for the support of their theory (Snowden, 2008). Doubt in the validity of the decreasing virulence theory began with novel disease discoveries during the 1970s; however, the tipping point arrived in 1983 with the discovery of the Human Immunodeficiency Virus (Shilts, 1988). Successive epidemics occurred throughout the 1990’s. In 1991, an epidemic of cholera swept across Central and South America (WHO, 2007). The US “four-corner” region was struck by a mysterious severe pulmonary illness which rapidly killed 83 individuals in 1993. Later identification of Hantavirus gave a name to the disease which had struck apprehension into the United States population (Epstein, 1995). Finally, with an epidemic of plague in India (1994) and the well publicized Ebola outbreak in Zaire during 1995, a clear epidemiological transition was occurring (Snowden, 2008); but what was occurring and why was it taking place?

Epidemiological Transition Theory: Model Expansion

Barrett and colleagues (1998), present a third epidemiological transition the Age of "Emerging and Re-emerging Infectious Diseases". Emerging diseases, defined by Davis and Lederberg (2000) are "diseases of infectious origin whose incidence in humans has increased within the past two decades or threatens to increase in the near future." The emerging and re-emerging infectious disease theory is based on the following three defining criteria: 1) numerous new diseases detected which are contributing to mortality, 2) increasing prevalence and incidence of diseases previously thought to be controlled, and 3) re-emerging diseases are developing anti-microbial resistance faster than new drugs to combat them can be developed.

The first criteria is easily observable as 31 emerging infectious diseases were identified from 1973-1995 (Lederberg, 1997). Currently, the United States National Institute of Allergy and Infectious Diseases (NIAID) lists 16 emerging and five re-emerging infectious diseases identified within the last two decades (Table 1A, NIAID, 2010). Defending the second criteria is the estimate that more people are infected with tuberculosis than at any time in human history (Hayward & Coker, 2008). Microbial resistance was first discovered in 1917 during the testing of the organic chemical Optochine ($C_{21}H_{28}N_2O_2$) in the treatment of pneumococcal pneumonia (Moellering, 1995, as cited in Barrett *et al.,* 1998). Microbial resistance continues to be a major public health hazard seen in the estimated 19,000 deaths in the US during 2005 caused by strains of

Staphylococcus aureus resistant to commonly administered antibiotics (Klevens *et al.,* 2007).

As a result of highly publicized infectious disease outbreaks (e.g. ebola, necrotizing fasciitis (e.g. *Streptococcus pyogenes*), human immunodeficiency virus (HIV)) and food recalls as a result of contamination with *Escherichia coli* O157:H7, popular opinion may point towards an overwhelming increase in the incidence and prevalence of infectious diseases; however, is the infectious disease apprehension we are experiencing today simply a result of increased surveillance capabilities? Until 2008, no analytic study had been published which undertook this question in a systematic manner. Jones (2008) published a study in which he compiled data on infectious diseases and surveillance capabilities from 1940 to 2004. Results of the study show that infectious diseases have been emerging at a rate above expected increases due to improved surveillance coverage. Furthermore, infectious disease emergence was shown to peak in the 1980's, but emergence today still exceeds that in the 1940's (Jones, 2008).

Decreased funding for infectious disease public health programs during the 1960's and 1970's played a large role in the emergence and re-emergence of infectious diseases, but many other factors have significantly contributed. Cohen (2000) has presented a graphic representation of the risk factors which have contributed to infectious disease emergence and re-emergence (Figure 2A). These risk factors include changes to human behavior; demographics changes, of which population increase and urbanization are significant contributors;

technological changes; industry changes; environmental changes; travel, and more specifically mass travel; decreases in public health infrastructure; and microbial adaptation (Cohen, 2000).

Table 1A. NIAID Global Emerging and Re-emerging Infectious Diseases: 1990-Present.

Emerging	Re-Emerging
Acanthamebiasis	Enterovirus 71
Australian bat lyssavirus	Clostridium difficile
Babesia, atypical	Mumps virus
Bartonella henselae	Streptococcus, Group A
Ehrlichiosis	Staphylococcus aureus
Encephalitozoon cuniculi	
Encephalitozoon hellem	
Enterocytozoon bieneusi	
Helicobacter pylori	
Hendra or equine morbilli virus	
Hepatitis C	
Hepatitis E	
Human herpesvirus 8	
Human herpesvirus 6	
Lyme borreliosis	
Parvovirus B19	

While the risk factors to increased infectious disease outcomes have been identified, the consequences on birth rates, death rates, and total population have yet to fully manifest as the emerging and re-emerging epidemiological transition is ongoing. Currently, public health efforts have been relatively successful in reducing the impact of acute infectious disease pandemics (e.g. SARS in 2003 and Influenza in 2009). However, a great disparity has arisen with regard to chronic infectious disease pandemics, such as HIV and tuberculosis.

These chronic infectious diseases, which have long latency and infectious periods, have increasingly affected all countries, but middle- and low- income countries have incurred the majority of the burden (Corbett, 2003). Ongoing vaccination development research and other similar public health endeavors help but do not fix the underlying causes of health disparity, being poverty, education, dietary needs and access to affordable healthcare (Snowden, 2008). Even with all the advances in health technology, Lederberg (1997) suggests that we are currently more susceptible to pandemic and communicable diseases threats than at any time in human history.

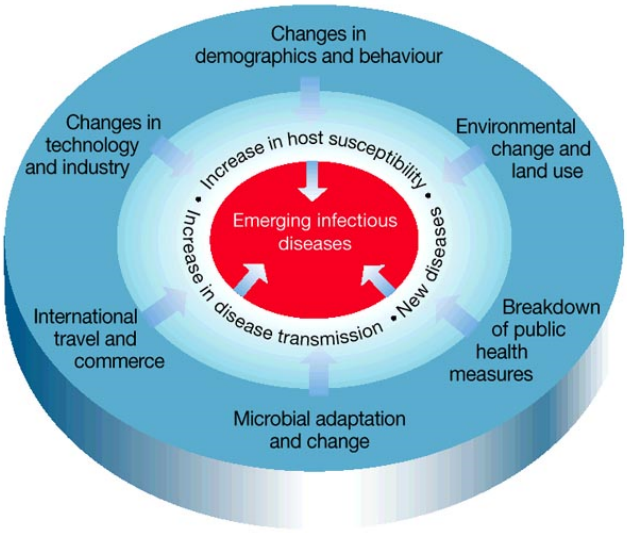


Figure 2A. "Changes in society, technology, environment and microorganisms are leading to increases in host susceptibility and/or disease transmission and the evolution of new or drug-resistant microorganisms" (Cohen, 2000).

WHO is predicting that non-infectious diseases will be responsible for little more than 5% of all global deaths by 2030 (WHO, 2008) (Figure 3A). When international organizations and governments decide to distribute aid and research funding the increasing prevalence on chronic diseases must be taken into consideration. Controlling chronic diseases must be met with the same, if not greater, monetary and cooperative effort seen in taking on infectious diseases in post-World War II. In the same breath, infectious diseases must not be forgotten, as history has already shown us their ability to emerge and re-emerge if public health infectious disease programs are de-funded. Unlike most chronic diseases, many infectious diseases have the potential to be eradicated by-well designed vaccines. Vaccine development should continue to be at the forefront of infectious disease funding. Understanding the biological pathway behind infectious disease pathogenesis is imperative for vaccine and treatment advancement. The effort to understand infectious disease pathogenesis must not be limited to funding of laboratory endeavors. Sponsorship of field and clinical epidemiology provides crucial evidence on the population effected. Epidemiology has aided in the discovery that diseases are not caused by "bad air", that HIV is not a disease limited to homosexuals, and that Severe Acute Respiratory Syndrome (SARS) is spread through direct or indirect transmission (Hempel, 2007; Shilts, 1988; PubMed Health, 2009).

Epidemiological studies, whether through surveillance or well-designed targeted studies, should not be thought of as a one-time undertaking to

comprehend an infectious disease as they have shown an uncanny ability to mutate and spread to novel geographic regions (Lowy, 2010). To fully understand a disease, epidemiological studies must encompass all potentially effected individuals in regards to place, but also time. The following section presents a historic review of dengue epidemiology.

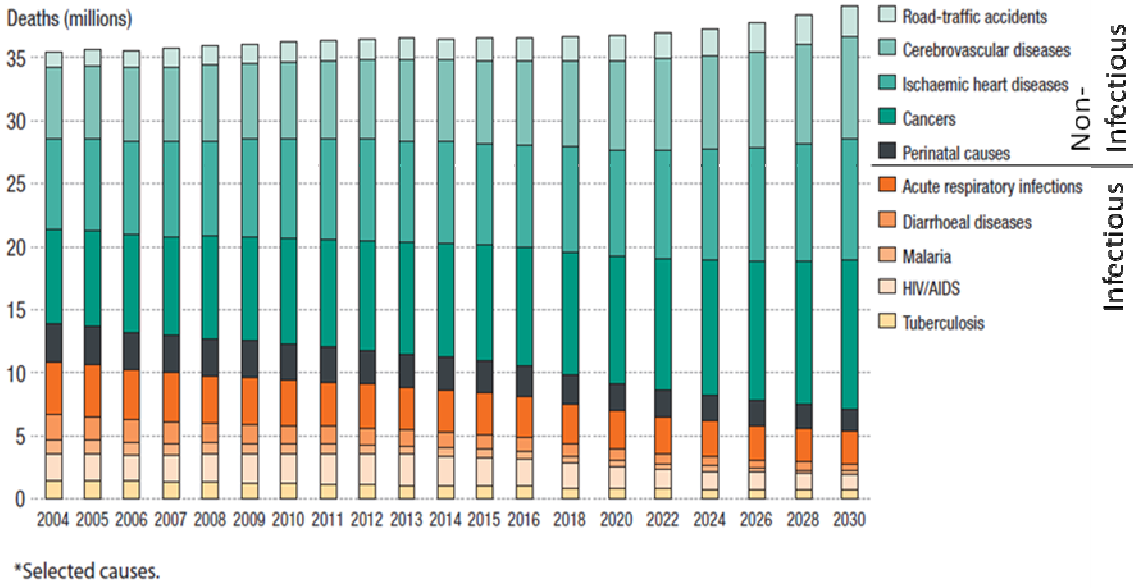


Figure 3A. The shift towards noncommunicable diseases and accidents as causes of death. (WHO, 2008) edited.

Appendix B: Primary Study Population Descriptive Tables

Table 1B. Dengue Manifestations in Source Study Participants by 2009 WHO Dengue Diagnostic Scheme from Hospital Ampang, 2010.[^]

	Non-Dengue		Without Warning Signs		With Warning Signs		Severe		Dengue Total	
Manifestation[*]	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Total Study Participants	22	8.0	27	9.8	222	80.4	5	1.8	254	92.0
Abdominal Pain	12	54.6	1	3.7 ‡	161	72.5 ¥	2	40.0	164	64.6
Arthraglia/Myaglia	17	77.3	20	74.1	184	82.9	3	60.0	207	81.5
Diarrhea	7	31.8	11	40.7	130	58.6	2	40.0	143	56.3
Hepatosplenomegaly	5	22.7	2	7.4	65	29.3	3	60.0	70	27.6
Nausea/Vomiting	15	68.2	10	37.0	203	91.4 ‡¥	5	100.0 ‡¥	218	85.8
Postural Giddiness	6	27.3	9	33.3	86	38.7	2	40.0	97	38.2
Rash	1	4.6	3	11.1	39	17.6	4	80.0	46	18.1
Retro-orbital/Headache	10	45.5	16	59.3	112	50.5	2	40.0	130	51.2
Viral Hepatitis	3	13.6	16	59.3 ‡	162	73.0 ‡	2	40.0	180	70.9 ‡
Bleeding Tendency (Any)[#]	5	22.7	2	7.4	62	27.9	3	60.0	67	26.4

[^] Fisher's exact test (2-tails) with Bonferroni correction.

* Other: Ascites (n=2), Pleural Effusion (n=7), & Petechiae/Bruises (n=4)

[#] Includes: epistaxis, gum provocative & spontaneous, hemetemesis, melena, menorrhagia, retinal and subconjunctival.

'Non-Dengue' control with level of significance: † ≤0.05 ; ‡ ≤0.01

'Without Warning' control with level of significance: ¥ ≤0.05

Table 2B. Blood Profile of Source Study Participants by 2009 WHO Dengue Diagnostic Scheme from Hospital Ampang, 2010.^

Profile Component	Non-Dengue		Without Warning		With Warning		Severe		Total Dengue	
	n	Avg. ± S.D.	*n*	Avg. ± S.D.	*n*	Avg. ± S.D.	*n*	Avg. ± S.D.	Avg. ± S.D.	
Hemoglobin[#]										
Febrile	13	13.1 ± 1.5	18	14.3 ± 1.4	154	13.9 ± 1.8	4	13.0 ± 2.3	13.9 ± 1.7	
Defervescence	20	13.4 ± 1.8	25	13.7 ± 1.6	219	13.6 ± 1.8	5	13.8 ± 1.7	13.6 ± 1.8	
Convalescence	16	13.0 ± 1.9	23	14.0 ± 1.4	201	13.7 ± 1.7	5	12.6 ± 1.9	13.7 ± 1.7	
Hematocrit[&]										
Febrile	14	39.2 ± 4.1	15	43.3 ± 3.5 †	155	42.1 ± 4.9	4	39.4 ± 7.7	42.1 ± 4.8	
Defervescence	20	40.0 ± 5.2	26	41.2 ± 5.9	221	41.0 ± 5.5	5	41.5 ± 5.6	41.0 ± 5.4	
Convalescence	17	38.2 ± 5.1	22	42.0 ± 5.9	202	41.5 ± 4.7 †	5	38.8 ± 5.8	41.5 ± 4.9	†
Platelet[*]										
Febrile	14	107.9 ± 58.3	18	85.9 ± 52.9	155	77.1 ± 42.4 †	4	107.3 ± 8.4	78.7 ± 43.2	
Defervescence	20	96.7 ± 44.0	26	70.1 ± 36.2	222	61.3 ± 43.2 ‡	5	82.8 ± 37.1	62.6 ± 42.4	‡
Convalescence	17	198.2 ± 71.8	23	209.7 ± 118.7	202	228.6 ± 140.9	5	324.4 ± 191.2	228.8 ± 140.2	
Total White Cell[*]										
Febrile	14	5.7 ± 3.1	18	3.6 ± 1.6	155	3.5 ± 1.9 ‡	4	5.7 ± 3.3	3.6 ± 1.9	
Defervescence	20	5.4 ± 3.2	26	4.2 ± 2.1	220	5.0 ± 2.4	5	6.7 ± 4.9	4.9 ± 2.4	
Convalescence	17	6.5 ± 2.2	22	6.3 ± 2.2	202	7.0 ± 5.4	5	7.1 ± 2.8	6.9 ± 5.1	
Neutrophil[*]										
Febrile	12	4.1 ± 2.3	12	1.5 ± 0.8 ‡	142	1.9 ± 1.4 ‡	4	4.4 ± 3.6	1.9 ± 1.5	†
Defervescence	17	3.7 ± 3.3	22	1.3 ± 0.8 †	218	1.7 ± 1.3 ‡	5	3.7 ± 4.3	1.7 ± 1.4	
Convalescence	15	3.9 ± 1.7	22	2.5 ± 1.0 †	210	2.9 ± 1.7	5	3.2 ± 1.9	2.8 ± 1.6	†
Lymphocyte[*]										
Febrile	12	1.5 ± 1.7	12	1.2 ± 0.7	142	1.0 ± 0.6	4	0.7 ± 0.7	1.0 ± 0.6	
Defervescence	17	1.4 ± 0.5	22	1.8 ± 1.1	218	1.9 ± 1.0	5	1.5 ± 1.0	1.9 ± 1.0†	
Convalescence	15	2.5 ± 1.2	22	2.8 ± 0.9	210	2.7 ± 1.0	5	2.9 ± 1.1	2.7 ± 1.0	
Monocyte[*]										
Febrile	11	0.6 ± 0.3	10	0.7 ± 0.5	125	0.6 ± 0.7	4	0.5 ± 0.1	0.6 ± 0.7	
Defervescence	17	0.6 ± 0.3	21	1.1 ± 0.9	199	1.2 ± 0.9	5	0.9 ± 0.3	1.2 ± 0.9	‡
Convalescence	12	0.7 ± 0.4	18	0.9 ± 0.9	182	0.9 ± 0.7	5	0.7 ± 0.4	0.9 ± 0.7	
Eosinophil[*]										
Febrile	11	0.03 ± 0.07	10	0.03 ± 0.09	125	0.01 ± 0.06	4	<0.0 ± <0.0	0.01 ± 0.06	
Defervescence	17	0.06 ± 0.09	21	0.03 ± 0.08	199	0.05 ± 0.10	5	0.02 ± 0.05	0.05 ± 0.09	
Convalescence	12	0.13 ± 0.11	18	0.12 ± 0.14	182	0.15 ± 0.17	5	0.06 ± 0.09	0.14 ± 0.16	
Basophil[*]										
Febrile	11	<0.00 ± <0.00	10	<0.00 ± <0.00	125	0.01 ± 0.03	4	<0.00 ± <0.00	0.01 ± 0.03	
Defervescence	17	<0.00 ± <0.00	21	<0.00 ± <0.00	199	0.01 ± 0.03	5	<0.00 ± <0.00	0.01 ± 0.03	
Convalescence	12	<0.01 ± 0.03	18	0.01 ± 0.03	182	0.02 ± 0.04	5	0.02 ± 0.05	0.02 ± 0.04	

^ Independent student t-test with Bonferroni correction. 'Non-Dengue' control group.
Level of Significance: † ≤0.05; ‡ ≤0.01
[*] (x10^9) ; [&] % ; [#] (g/dL)
Note: Significant associations were not detected between Without & With Warning groups.

Table 3B. Renal Profile of Source Study Participants by 2009 WHO Dengue Diagnostic Scheme from Hospital Ampang, 2010.[^]

	Non-Dengue		Without Warning		With Warning		Severe		Total Dengue
Profile Component	*n*	Avg. ± S.D.	*n*	Avg. ± S.D.	*n*	Avg. ± S.D.	*n*	Avg. ± S.D.	Avg. ± S.D.
Urea[*]									
Febrile	11	5.3 ± 3.2	11	3.9 ± 1.7	132	3.2 ± 1.6 ‡	3	3.3 ± 0.8	3.2 ± 1.6
Defervescence	17	4.5 ± 3.7	22	2.5 ± 1.2	215	2.4 ± 1.0 ‡	5	2.9 ± 1.4	2.5 ± 1.1
Convalescence	14	4.1 ± 1.8	17	2.8 ± 1.1	173	2.5 ± 1.1 ‡	5	3.1 ± 1.4	2.5 ± 1.1 †
Sodium[*]									
Febrile	11	136.3 ± 7.3	11	135.5 ± 3.9	132	136.2 ± 3.5	3	133.3 ± 4.5	136.1 ± 3.5
Defervescence	17	138.4 ± 4.3	22	140.4 ± 4.8	215	139.1 ± 2.9	5	138.0 ± 2.4	139.2 ± 3.1
Convalescence	14	139.9 ± 2.6	17	140.6 ± 2.6	172	139.8 ± 2.6	5	140.6 ± 2.1	139.9 ± 2.6
Potassium[*]									
Febrile	10	3.9 ± 0.4	11	3.5 ± 0.2 †	132	3.6 ± 0.4	3	4.4 ± 1.0 †	3.6 ± 0.4
Defervescence	17	3.7 ± 0.4	22	3.7 ± 0.4	214	3.7 ± 0.5	5	3.7 ± 0.4	3.7 ± 0.5
Convalescence	14	3.7 ± 0.4	17	3.8 ± 0.3	173	4.0 ± 2.3	5	3.8 ± 0.4	4.0 ± 2.1
Chloride[*]									
Febrile	11	102.7 ± 7.0	11	102.2 ± 4.6	132	101.6 ± 4.4	3	97.0 ± 6.1	101.6 ± 4.4
Defervescence	17	105.6 ± 4.7	22	107.2 ± 5.3	215	105.8 ± 4.0	5	102.6 ± 5.6	105.8 ± 4.2
Convalescence	14	106.0 ± 4.2	17	105.1 ± 4.2	173	104.3 ± 3.8	5	103.0 ± 1.2	104.3 ± 3.8
Creatinine[&]									
Febrile	11	94.1 ± 49.7	11	94.6 ± 27.0	132	74.1 ± 23.3 ¥	3	92.0 ± 22.6	76.0 ± 24.1
Defervescence	17	84.7 ± 52.9	21	66.6 ± 18.6	215	63.3 ± 17.1 ‡	5	75.8 ± 35.4	63.8 ± 17.7
Convalescence	14	82.1 ± 29.0	17	69.8 ± 21.8	173	62.1 ± 16.3 ‡	5	60.8 ± 33.0	62.7 ± 17.0

[^] Independent student t-test with Bonferroni correction.
† ≤0.05 level of significance with 'Non-Dengue' control group.
¥ ≤0.05 level of significance with 'Without Warning' control group.
[*] mmol/L ; [&] umol/L

Table 4B. Liver Profile of Source Study Participants by 2009 WHO Dengue Diagnostic Scheme from Hospital Ampang, 2010.[^]

	Non-Dengue		Without Warning		With Warning			Severe		Total Dengue	
Profile Component	*n*	Avg. ± S.D.	*n*	Avg. ± S.D.	*n*	Avg. ± S.D.		*n*	Avg. ± S.D.	Avg. ± S.D.	
Total Bilirubin[*]											
Febrile	11	34.0 ± 52.9	11	10.6 ± 8.0	132	8.1 ± 4.9	‡	3	12.7 ± 11.7	8.4 ± 5.4	
Defervescence	15	14.0 ± 12.1	22	7.3 ± 3.8	215	8.7 ± 4.8	‡	5	12.8 ± 8.3	8.6 ± 4.9	
Convalescence	14	15.0 ± 11.8	21	8.6 ± 3.5	210	10.1 ± 5.6	‡	5	11.8 ± 7.3	10.0 ± 5.5	
Alkaline Phosphatase[&]											
Febrile	11	71.7 ± 27.4	11	82.3 ± 70.5	131	66.8 ± 31.4		3	68.7 ± 13.8	68.0 ± 35.4	
Defervescence	15	91.7 ± 82.9	22	56.9 ± 34.0	215	64.6 ± 35.3	†	5	56.2 ± 15.2	63.7 ± 34.9	
Convalescence	14	92.8 ± 60.2	21	72.2 ± 55.2	210	73.9 ± 36.9		5	62.8 ± 16.0	73.6 ± 38.5	
Total Protein[#]											
Febrile	11	65.3 ± 7.9	11	66.1 ± 7.4	131	67.4 ± 8.3		3	73.7 ± 4.2	67.5 ± 8.2	
Defervescence	15	59.9 ± 4.2	22	62.0 ± 4.8	215	61.9 ± 6.9		5	66.8 ± 9.1	62.0 ± 6.8	
Convalescence	14	67.1 ± 9.9	21	72.7 ± 7.1	210	73.2 ± 8.7	†	5	74.0 ± 6.8	73.2 ± 8.5	†
Albumin[#]											
Febrile	11	34.6 ± 5.1	11	38.4 ± 4.5	131	37.9 ± 4.5		3	39.0 ± 1.0	38.0 ± 4.4	
Defervescence	15	32.1 ± 5.0	22	34.2 ± 3.5	215	34.1 ± 3.9		5	35.4 ± 7.3	34.2 ± 4.0	
Convalescence	14	35.9 ± 8.3	21	40.6 ± 3.5	210	40.5 ± 4.9	†	5	39.6 ± 6.1	40.5 ± 4.8	
Globulin[#]											
Febrile	11	30.7 ± 5.8	11	27.7 ± 4.9	131	29.5 ± 6.3		3	34.7 ± 4.5	29.5 ± 6.2	
Defervescence	15	27.9 ± 4.1	22	27.7 ± 4.8	215	27.7 ± 4.9		5	31.4 ± 4.2	27.8 ± 4.9	
Convalescence	14	31.3 ± 4.8	21	32.0 ± 5.7	210	32.7 ± 6.4		5	34.4 ± 4.3	32.7 ± 6.3	
Alanine Aminotransferase[&]											
Febrile	11	53.6 ± 47.9	11	77.4 ± 47.7	131	90.6 ± 84.0		3	43.0 ± 9.0	88.6 ± 81.1	
Defervescence	15	75.3 ± 77.4	22	95.0 ± 95.4	215	115.8 ± 112.7		5	67.2 ± 42.7	112.9 ± 110.4	
Convalescence	14	51.9 ± 27.8	21	77.9 ± 55.9	210	90.5 ± 72.5		5	94.2 ± 96.9	89.5 ± 71.5	

[^] Independent student t-test with Bonferroni correction. 'Non-Dengue' control group.
Level of Significance: † ≤0.05; ‡ ≤0.01
[*] umol/L ; [&] U/L ; [#] (g/L)
Note: Significant associations were not detected between Without & With Warning groups.

Table 5B. Vital Statistic Profile of Source Study Participants by 2009 WHO Dengue Diagnostic Scheme from Hospital Ampang, 2010.[^]

	Non-Dengue		Without Warning		With Warning		Severe		Total Dengue
Profile Component	*n*	Avg. ± S.D.	n	Avg. ± S.D.	n	Avg. ± S.D.	*n*	Avg. ± S.D.	Avg. ± S.D.
Temperature (˚C)									
Febrile	15	38.9 ± 0.7	18	38.6 ± 0.8	148	38.7 ± 0.8	4	39.0 ± 0.9	38.7 ± 0.8
Defervescence	18	37.0 ± 0.3	22	37.1 ± 0.2	186	37.1 ± 0.3	2	36.7 ± 0.4	37.1 ± 0.3
Convalescence	4	37.0 ± 0.6	4	37.2 ± 0.3	7	36.9 ± 0.1	1	37	37.0 ± 0.3
Systolic Blood Pressure[#]									
Febrile	15	123.8 ± 30.2	18	122.4 ± 16.9	148	121.7 ± 15.2	4	99.0 ± 18.1	121.5 ± 17.2
Defervescence	18	124.4 ± 20.3	21	115.7 ± 13.2	173	118.2 ± 12.5	2	118.0 ± 25.5	118.5 ± 13.5
Convalescence	4	109.8 ± 11.6	5	109.0 ± 5.5	5	117.4 ± 19.2	0	-	112.2 ± 13.0
Diastolic Blood Pressure[#]									
Febrile	15	73.3 ± 15.7	18	73.9 ± 10.9	148	75.2 ± 12.0	4	60.0 ± 17.2	74.6 ± 12.4
Defervescence	18	75.9 ± 7.7	20	72.6 ± 11.8	173	75.8 ± 9.7	2	67.0 ± 9.9	75.4 ± 9.8
Convalescence	4	75.0 ± 7.0	5	72.0 ± 9.3	5	66.8 ± 5.1	0	-	71.0 ± 7.6
Pulse Rate[*]									
Febrile	15	96.3 ± 19.6	18	93.8 ± 17.6	147	93.2 ± 16.4	4	106.3 ± 9.1	93.8 ± 16.7
Defervescence	0	-	0	-	0	-	0	-	-
Convalescence	4	75.5 ± 9.0	4	80.8 ± 17.4	5	79.6 ± 7.2	0	-	78.7 ± 10.9

[^] Independent student t-test with Bonferroni correction.

[*] mmHg ; [#] beats per minute

Note: Significant associations were not detected with control group as 'Non-Dengue' or 'Without Warning.'

Appendix C: SAS® Code for Statistical Analysis

```
*************************************************************************
*********************
SAS Program for Benjamin Klekamp's thesis: 2011
 Defended May 17th, 2011
 Uses SAS dataset: Thesis_Final
*************************************************************************
*********************;
*Importing the datset;
libname perm "C:\Users\Desktop\Ben\Thesis\" ;
Data data1 ; Set perm.thesis_final ; run;

*Hemagglutination inhibition results - collaspsing into primary and
secondary infection
*Only outputs primary and secondary infections
*HemIn - is hemagglutination inhibition (exposure) variable (binary
(1&2)) ;
Data data2 ; Set data1 ;
if HI = 'p2' then HemIn = 2 ;
else if HI = '2' then HemIn = 2 ;
else if HI = '1' then HemIn = 1 ;
else if HI = 'p1' then HemIn = 1 ;
else HemIn = 0 ;
if HemIn ne 0 then output;
run;

*Some participants did not have a blood profile conducted during
defervescence phase of illness
*Absolute Monocyte Count (part of the blood profile) is the outcome
variable
*mono2 - was the variable representing absolute monocyte count during
defervescence
*Only those participants who have a recorded value for mono2 are
output;
Data Data3; Set Data2;
if mono2 ne . then output; run;

*Creating a variable to be used in the secondary aim analysis
*HEM_VL = absence (0) or presence (1) of hemorrhage and/or vascular
leakage;
Data Data4; Set Data3;
VL=0;
Hem=0;                                                  *VL = vascular
leakage;
HEM_VL=0;                                            *HEM = hemorrhage;
if HCT1 ne . and HCT2 ne . then do; *HCT1 = febrile hematocrit;
HCTp = (HCT1 - HCT2)/HCT1*100;             *HCT2 = defervescence
hematocrit;
if HCTp ge 20 then VL = 1;                 *HCTp = hematocrit percent
change;
if HCTp le -20 then VL = 1;
```

```
if PE = 1 then VL = 1 ;                    *PE = absence (0) or presence
(1) of pleural effusion;
if ASC = 1 then VL = 1 ;                   *ASC = absence (0) or
presence (1) of ascites;
if SBP2 lt 90 then VL = 1;                 *SBP2 = systolic blood
pressure at defervescence;
if ((SBP2-DBP2)) le 20 then VL = 1; *DBP2 = distolic blood pressure at
defervescence;
end;

if
SGB  = 1 or   /*Spontaneous gum bleeding*/
EPIX = 1 or   /*Epistaxis*/
MNG  = 1 or   /*Menorrhagia*/
HEMT = 1 or   /*Hemetemesis*/
SCH  = 1 or   /*Subconjunctival haemorrhage*/
RH   = 1 or   /*Retinal haemorrhage*/
MEL  = 1      /*melena*/
then Hem = 1 ;

if Hem = 1 or VL = 1 then HEM_VL = 1; run;

*Age of patient is calculated;
*Duration between first symptom and admission is calculated
(Tadmission);
*Duration of Hospitlization is calculated (Tdischarge);
*Classifying age into youth - adult, non-youth - elderly;
*Classifying distance between patient residence and Hospital Ampang by
12.5km;
*Combining manifestation variables for analysis ;
Data Data5; Set Data4;
AGE=(DATE2-DOB)/365.25; *Date2 = date of defervescence blood draw;
*DOB = Date of birth;

Tadmission=DOA - FEVD ; *DOA= Date of admission;  *FEVD= Date of first
sysmptom;
Tdischarge=DOD - DOA ;  *DOD= Date of Discharge;

if Age ge 15 and Age lt 25 then Age1 = 1;
else if Age ge 25 and Age lt 35 then Age1 = 2;
else if Age ge 35 and Age lt 45 then Age1 = 3;
else if Age ge 45 then Age1 = 4 ;

if kilometers le 12.5 then KM = 0 ;
else km = 1;

if arth = 1 or mya = 1 then MYAR = 1 ; *arth= arthralgia; *mya=
myalgia;
      else myar = 0;
if spleen = 1 or liver = 1 then HEPspl = 1 ; *spleen= Splenomegaly;
*liver= Hepatomegaly;
      else hepspl = 0 ;
if nau = 1 or vom = 1 then nauvom = 1 ; *nau = nausea;  *vom=vomitting;
      else nauvom = 0;
```

```
run;

*Creation of a final permenent dataset to work off;
Data perm.FINAL ; Set data5 ; run;

*************************************************************************
********;
*ANALYSIS OF THE DATASET;
*************************************************************************
********;

* Creating time to admission and time to discharge values;
proc means data= perm.final;
var Tadmission Tdischarge; run;
proc sort data=perm.final; by hemin; run;
proc means data= perm.final;
by hemin;
var Tadmission Tdischarge;
run;
proc sort data=perm.final; by hem_vl; run;
proc means data= perm.final;
by hem_vl;
var Tadmission Tdischarge;
run;

*Table 1 was copied from previous report;

*Creating values for Table 2;
proc freq data = perm.final;
table (sex race age1 km)*HemIn; run;
PRoc freq data= perm.final;
table (sex race age1 km)*Hem_vl ; run;

*Creating Table 3;
proc freq data = perm.final;
table (dm HPT ckd ihd ccf)*HemIn; run;
proc freq data = perm.final;
table (dm HPT ckd ihd ccf)*hem_vl; run;

*Creating Table 4 ;
proc freq data = perm.final;
table (AP MYAR dia HEPspl nauvom pgid rash cereb hepa hem vl)*HemIn;
run;
proc freq data = perm.final;
table (AP MYAR dia HEPspl nauvom pgid rash cereb hepa)*Hem_vl; run;

* Creating Table 5;
proc means data = perm.final;
var mono2 plt2 hct2 ; run;
proc sort data=perm.final; by hemin; run;
proc means data = perm.final;
by hemin;
var mono2 plt2 hct2 ; run;
proc sort data=perm.final; by hem_vl; run;
```

```
proc means data = perm.final;
by hem_vl;
var mono2 plt2  hct2 ; run;

****Assessing Distance for confounding;
PRoc freq data=perm.final;
table Hemin*km / chisq;
run;
proc sort data= perm.final; by km; run;
proc means data=perm.final t prt mean std;
var mono2;
by km;
run;
PRoc freq data=perm.final;
table Hem_vl*km / chisq;
run;

*Creating Table 6 - linear regression;
proc reg  data = perm.final  ;
model  mono2 =  HemIN age sex race ;
output p=p r=r out=LinReg_base;
attrib _all_ label=' ';
title'Linear - Age continuous - without distance';
run; quit;

axis1 order=-2 to 3 by 1;
axis2 order=0.3 to 1.7 by 0.2;
title'Linear - Age continuous - without distance';
Proc gplot data=LinReg_base;
plot r*p / vaxis=axis1 haxis=axis2;
run; quit;

*Table 7 ;
proc reg  data = perm.final  ;
model  mono2 =  HemIN age sex race km ;
output p=p r=r out=LnReg_KM;
attrib _all_ label=' ';
title'Linear - Age continuous - with distance';
run; quit;

axis1 order=-2 to 3 by 1;
axis2 order=0.3 to 1.7 by 0.2;
title 'Linear - Age continuous - with distance';
Proc gplot data=LnReg_KM;
plot r*p / vaxis=axis1 haxis=axis2;
run; quit;
************************************************;
*Table 8;
proc reg  data = perm.final  ;
model  mono2 =  HemIN age1 sex race ;
output p=p r=r out=LnReg_Age3;
attrib _all_ label=' ';
title'Linear - Age by 10 years - without distance';
run; quit;
```

```
axis1 order=-2 to 3 by 1;
axis2 order=0.3 to 1.7 by 0.2;
title'Linear - Age by 10 years - without distance';
Proc gplot data=LnReg_Age3;
plot r*p / vaxis=axis1 haxis=axis2;
run; quit;

*Table 9;
proc reg  data = perm.final  ;
model  mono2 =  HemIN age1 sex race km ;
output p=p r=r out=LnReg_Age3km;
attrib _all_ label=' ';
title'Linear - Age by 10 years - with distance';
run; quit;

axis1 order=-2 to 3 by 1;
axis2 order=0.3 to 1.7 by 0.2;
title'Linear - Age by 10 years - with distance';
Proc gplot data=LnReg_Age3km;
plot r*p / vaxis=axis1 haxis=axis2;
run; quit;

************************************************;
*Creating Table 10 - logistic regression;
proc logistic  data = perm.final  ;
class    sex  race (descending) hemin / param =ref ref =last     ;
model  hem_VL (EVENT='1')=  mono2 age sex  race hemin   / influence
lackfit;
output out=LogReg_base p=p reschi=PR;
attrib _all_ label=' ';
title'Logistic - Age continuous - without distance';
run;
title'Logistic - Age continuous - without distance';
Proc gplot data=LogReg_base;
plot pr*p  ;
run; quit;

*Table 11;
proc logistic  data = perm.final  ;
class  sex  race (descending) hemin km (descending)  / param =ref ref
=last   ;
model  hem_VL (EVENT='1')=  mono2 age sex  hemin race km / influence
lackfit ;
output out=LogReg_km p=p reschi=PR;
attrib _all_ label=' ';
title'Logistic - Age continuous - with distance';
run;
title'Logistic - Age continuous - with distance';
Proc gplot data=LogReg_km;
plot pr*p  ;
run; quit;
************************************************;
*Table 12;
```

```
proc logistic  data = perm.final  ;
class  age1 (descending) sex  race (descending) hemin km (descending)
/ param =ref ref =last   ;
model  hem_VL (EVENT='1')=  mono2 age1 sex  hemin race / influence
lackfit ;
output out=LogReg_Age1 p=p reschi=PR;
attrib _all_ label=' ';
title'Logistic - Age by 10 years - without distance';
run;
title'Logistic - Age by 10 years - without distance';
Proc gplot data=LogReg_Age1;
plot pr*p  ;
run; quit;

*Table 13;
proc logistic  data = perm.final  ;
class  age1 (descending) sex  race (descending) hemin km (descending)
/ param =ref ref =last   ;
model  hem_VL (EVENT='1')=  mono2 age1 sex  hemin race km / influence
lackfit ;
output out=LogReg_Age1km p=p reschi=PR;
attrib _all_ label=' ';
title'Logistic - Age by 10 years - with distance';
run;
title'Logistic - Age by 10 years - with distance';
Proc gplot data=LogReg_Age1km;
plot pr*p  ;
run; quit;
```

ABOUT THE AUTHOR

Benjamin Klekamp graduated from James Madison Memorial High School in Madison, Wisconsin in 2002. He graduated from Lawrence University in Appleton, Wisconsin with a Bachelor in Arts in Biology in 2006. Following graduation, Ben was employed as a Production Scientist in the Nucleic Acid Chemistry Department at Promega Corporation in Madison, Wisconsin until July 2008. In the fall of 2008, Ben began a Masters in Science in Public Health majoring epidemiology and Certificate of Infection Control at the College of Public Health, University of South Florida in Tampa, Florida. Ben spent from June 2010 to February 2011 in Kuala Lumpur, Malaysia working as a volunteer graduate assistant under Professor Dr. Shamala Devi Sekaran of the Department of Medical Biology, Faculty of Medicine, University of Malaya. After graduating from the University of South Florida in August 2011, Ben obtained employment with the Florida Department of Health as a Fellow in the Epidemic Intelligence Service (FL-EIS) serving in Orange and Osceola counties.